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The F-box: a new motif for ubiquitin dependent proteolysis in cell cycle regulation and signal transduction

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Abstract

The ubiquitin system of intracellular protein degradation controls the abundance of many critical regulatory proteins. Specificity in the ubiquitin system is determined largely at the level of substrate recognition, a step that is mediated by E3 ubiquitin ligases. Analysis of the mechanisms of phosphorylation directed proteolysis in cell cycle regulation has uncovered a new class of E3 ubiquitin ligases called SCF complexes, which are composed of the subunits Skp1, Rbx1, Cdc53 and any one of a large number of different F-box proteins. The substrate specificity of SCF complexes is determined by the interchangeable F-box protein subunit, which recruits a specific set of substrates for ubiquitination to the core complex composed of Skp1, Rbx1, Cdc53 and the E2 enzyme Cdc34. F-box proteins have a bipartite structure — the shared F-box motif links F-box proteins to Skp1 and the core complex, whereas divergent protein–protein interaction motifs selectively bind their cognate substrates. To date all known SCF substrates are recognised in a strictly phosphorylation dependent manner, thus linking intracellular signalling networks to the ubiquitin system. The plethora of different F-box proteins in databases suggests that many pathways will be governed by SCF-dependent proteolysis. Indeed, genetic analysis has uncovered roles for F-box proteins in a variety of signalling pathways, ranging from nutrient sensing in yeast to conserved developmental pathways in plants and animals. Moreover, structural analysis has revealed ancestral relationships between SCF complexes and two other E3 ubiquitin ligases, suggesting that the combinatorial use of substrate specific adaptor proteins has evolved to allow the regulation of many cellular processes. Here, we review the known signalling pathways that are regulated by SCF complexes and highlight current issues in phosphorylation dependent protein degradation.

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1. Overview: phosphorylation directed proteolysis

Protein phosphorylation is a prevalent mechanism for modulating protein–protein interactions, which in turn control many aspects of cellular behaviour. The interaction of SH2 domains with tyrosine phosphorylated proteins in growth factor signalling pathways is perhaps the best understood phosphorylation dependent protein–protein interaction (Pawson, 1995). Serine/threonine kinases also effectively control the formation of specific protein complexes, as for example, in the interaction of 14-3-3 proteins with serine phosphorylated proteins. It also appears that serine phosphorylation creates a docking site for WW domains (Lu et al., 1999). In an analogous manner, phosphoinositide lipids serve to nucleate protein complexes at the cell membrane (Pawson and Scott, 1997). Recently, it has become apparent that phosphorylation on serine and threonine residues often marks proteins for rapid degradation by the ubiquitin proteolytic system. Phosphorylation in this context drives protein–protein interactions between the substrate and the enzymatic machinery that catalyses the covalent attachment of ubiquitin, which targets proteins for degradation by the 26*S* proteasome. Investigation of the protein degradation pathways that regulate cell cycle progression has uncovered a broad class of E3 enzymes, termed SCF complexes, that target a large number of substrates for phosphorylation dependent proteolysis.

2. The ubiquitin system

Rapid, specific intracellular protein degradation is instrumental in the control of cell cycle transitions, developmental programs, and responses to environmental signals. Most proteins with very short half-lives are degraded by the ubiquitin proteolytic system, which catalyses the covalent attachment of polyubiquitin chains to substrate proteins (Hershko and Ciechanover, 1998). Polyubiquitinated proteins are rapidly captured by the 26*S* proteasome, an abundant, self-compartmentalised protease particle (Baumeister et al., 1998). The 26*S* proteasome is composed of a 20*S* core particle (CP) and two 19*S* regulatory particles (RP). The CP is a cylindrical structure, flanked on each end by narrow channels that lead to a central lumen, which is laced with active protease sites. The RPs cap each end of the CP and regulate translocation of substrates into the lumen. The RP can be further divided into a base subcomplex, which contains ATPase subunits presumed to unfold and thread substrates into the lumen, and a lid subcomplex, which contains polyubiquitin binding subunits and de-ubiquitinating enzymes (Lam et al., 1997; Glickman et al., 1998). Proteasomes account for about 1% of total cellular protein, and the mobility of proteasomes in the cytosolic and nuclear compartments is essentially diffusion limited (Reits et al., 1997). Although the fate of most proteins targeted to the proteasome is destruction into short peptides of 8–10 amino acids in length, some proteins, such as the NFκB precursor, undergo limited proteolytic processing to generate mature proteins (Maniatis, 1999).

Ubiquitin conjugation occurs via a cascade of ubiquitin-transferase reactions, first resolved in a rabbit reticulocyte system by Hershko et al. (1983). Ubiquitin is activated for conjugation in an ATP dependent manner by formation of a thioester linkage between the C-terminal glycine residue of ubiquitin and a cysteine residue on an E1 ubiquitin activating enzyme.

Ubiquitin is then transesterified onto a cysteine residue of an E2 ubiquitin conjugating enzyme, and finally, through the action of an E3 ubiquitin ligase, linked in an isopeptide bond configuration to a lysine residue on the substrate protein. In many cases, the E3 participates directly in the transferase reaction, forming an intermediate thioester with ubiquitin (Scheffner et al., 1995). In other instances, the E3 juxtaposes the substrate and the E2 enzymes allowing direct transfer of ubiquitin from the E2 to the substrate, but the E3 does not itself form ubiquitin adducts (Hershko et al., 1983). In general, enzyme diversity increases down the ubiquitin transferase cascade. In yeast, a single E1 enzyme activates thirteen E2 enzymes that in turn couple to a large, but unknown, number of E3 enzymes (Hershko and Ciechanover, 1998). E1 and E2 enzymes are easily recognised because of their high degree of sequence conservation, whereas the few characterised E3 enzymes are structurally divergent, a characteristic that has hindered their identification in databases (Varshavsky, 1997). Ubiquitin metabolism is dynamic in that a large class of deubiquitinating enzymes probably serve to disassemble ubiquitin chains on substrates (Hochstrasser, 1996). In addition, a recently described 'E4' activity appears to act as a processivity factor for ubiquitin chain assembly on some substrates (Koegl et al., 1999). Finally, the ubiquitin system interacts with other conjugation systems that modify proteins by divergent ubiquitin related proteins (Hochstrasser, 1998).

At present, four unrelated classes of E3 enzymes have been identified, each having specificity for particular types of substrates. The archetypal E3 ubiquitin ligase, E3α, targets proteins by the 'N-end rule' pathway, in which the N-terminal amino acid of the substrate dictates recognition (Varshavsky, 1997). A second family of E3 enzymes, the HECT domain proteins (for homology to E6-AP C-terminus), is exemplified by E6-AP, which complexes with the human papillomavirus E6 protein to target the tumour suppressor p53 for degradation (Scheffner et al., 1993). Other HECT domain E3 enzymes include Rsp5, Nedd4 and Pub1, all of which are implicated in the degradation of specific membrane proteins and other targets (Hershko and Ciechanover, 1998). The specificity determinants in substrates recognised by HECT domain E3 enzymes are not well characterised. In one instance the WW domains of Nedd4 mediate binding to proline-rich PY motifs in the epithelial sodium channel in mammals. Deletion of the PY motifs results in stabilisation of the channel, and generates a hypertensive disorder called Liddle's syndrome (Staub et al., 1997). Another member of the HECT family, Rsp5, catalyses the mono-ubiquitination and subsequent lysosomal degradation of the Ste2 pheromone receptor (Terrell et al., 1998). In this instance, degradation is phosphorylation dependent (Hicke et al., 1998), suggesting that the WW domains of Rsp5 might selectively bind to serine phosphorylated sequences in the receptor C-terminus (Lu et al., 1999).

Two recently described types of E3 enzymes were identified because of their essential roles in the cell division cycle. The APC (for Anaphase Promoting Complex), or cyclosome, is a multisubunit E3 ubiquitin ligase complex that targets mitotic cyclins and other regulators of mitosis for destruction (Townsley and Ruderman, 1998). Recognition of substrates by the APC is mediated by a motif (present in all known APC substrates), termed the destruction box. The APC is active from the end of mitosis and throughout G1 phase, and thus the stability and abundance of APC substrates are dramatically affected during this window (Amon et al., 1994). The final class of E3 ubiquitin ligases, generically referred to as SCF complexes (for Skp1, Cdc53, F-box protein) mediates degradation of yeast G1 cyclins, Cdk inhibitors and

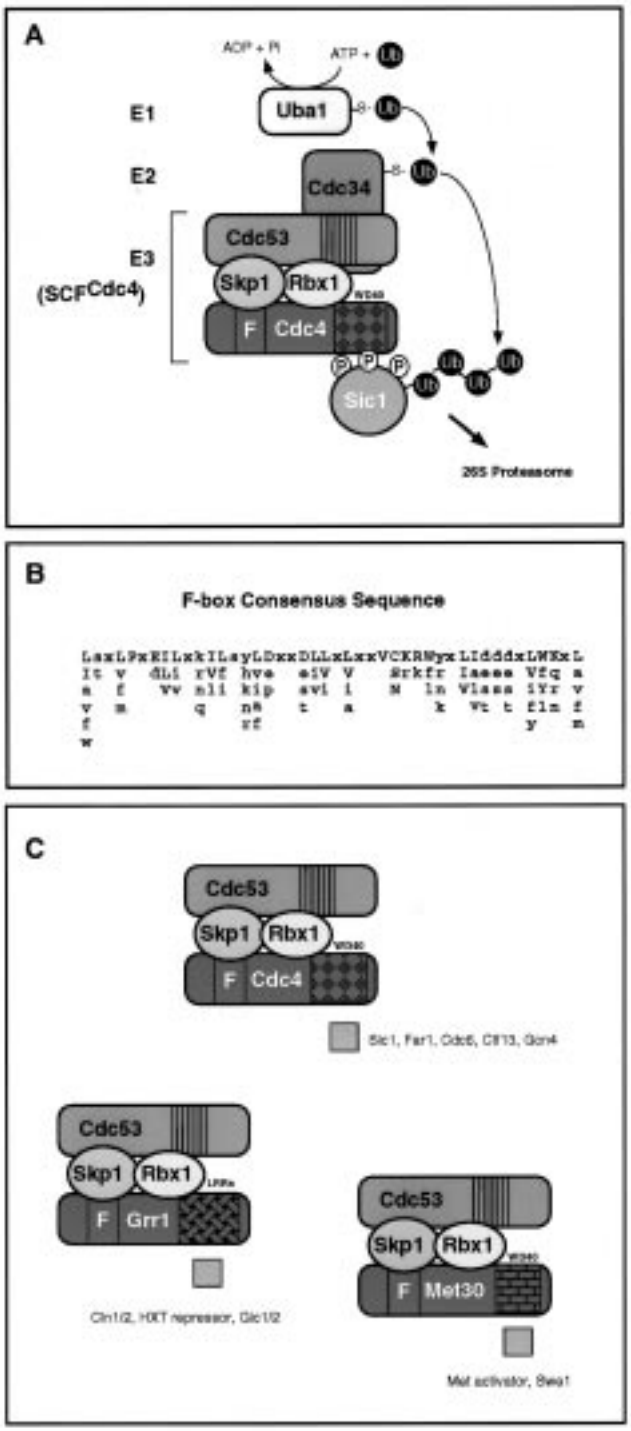

Fig. 1. (*caption opposite*).

many other proteins (Patton et al., 1998a). In contrast to the APC, SCF complexes appear to be constitutively active E3 enzymes that recognise and ubiquitinate only phosphorylated substrates. The SCF family thus links protein phosphorylation networks to proteolytic degradation. Despite their prominent role in the cell cycle, SCF complexes appear to regulate a huge number of other pathways by virtue of a multitude of substrate specific adaptor subunits called F-box proteins. Here, we review the SCF family of E3 enzymes with emphasis on both the known and postulated roles of SCF complexes in signal transduction pathways.

3. Lessons from the cell cycle: the SCF family of E3 ubiquitin ligases

The first SCF pathway was identified through analysis of the G1 to S-phase transition in the budding yeast *Saccharomyces cerevisiae*. In yeast, initiation of DNA replication requires mitotic cyclin (Clb)-cyclin-dependent kinase (Cdk) activity, which cannot develop until a Cdk inhibitor called Sic1 is destroyed (Schwob et al., 1994). Sic1 degradation in late G1 phase is triggered upon phosphorylation by G1 cyclin (Cln)-Cdk kinases and requires an E2 enzyme called Cdc34 (Schwob et al., 1994; Schneider et al., 1996; Tyers, 1996). A wealth of genetic and biochemical analysis has revealed that Cdc34 acts to ubiquitinate Sic1 in concert with an E3 ubiquitin ligase complex composed of the subunits Skp1, Cdc53, Rbx1 and Cdc4 (Fig. 1a) (Bai et al., 1996; Mathias et al., 1996; Willems et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; 1999; Kamura et al., 1999; Ohta et al., 1999). This class of E3 is generically referred to as an SCF complex, for the constituent proteins Skp1, Cdc53 and any one of a number of proteins that contain an F-box motif, such as Cdc4. Particular SCF complexes are designated by their associated F-box protein, for example SCF^{Cdc4}, SCF^{Grr1}, etc. Within SCF complexes, Skp1 binds to the F-box, which was first identified by alignment of three different Skp1 binding proteins (Fig. 1b) (Bai et al., 1996). Skp1 also interacts with the N-terminal region of Cdc53 and thereby connects Cdc4 to Cdc53 (Patton et al., 1998b). In turn, a conserved domain in the central region of Cdc53 links the E3 complex to Cdc34 (Patton et al., 1998b). Finally, Rbx1, a RING finger protein also known as Roc1 or Hrt1 appears to stabilise the E2/E3

Fig. 1. A) A complex of Skp1, Cdc53, Rbx1 and the F-box protein Cdc4 forms an E3 ubiquitin-protein ligase termed SCF^{Cdc4} in the budding yeast *S. cerevisiae*. Ubiquitin (Ub) is activated by forming a thioester bond with a cysteine residue of Uba1, an E1 enzyme, in a process requiring ATP. Ub is then transferred to the E2 enzyme Cdc34, and subsequently through the action of the E3 SCF^{Cdc4} conjugated to lysine residues of the target protein, in this case, the Cdk inhibitor Sic1. Polyubiquitinated proteins are recognised and degraded by the 26*S* proteasome. Sic1 is recruited to the E3 complex via a specific phosphorylation-dependent interaction with the C-terminal WD40 repeats of Cdc4. Cdc4 interacts with Skp1 via a conserved motif in Cdc4 termed the F-box. The N-terminal region of Cdc53 interacts with Skp1 while a conserved central region of Cdc53 interacts with Cdc34 (Patton et al., 1998b). B) An F-box consensus sequence generated from an alignment of 38 F-box-containing proteins. Highly conserved residues are capitalised (for details, see Patton et al., 1998a). C) Different F-box proteins interface with a core E3 complex via the F-box–Skp1 interaction to form a diverse family of SCF complexes. F-box proteins recruit specific substrates for ubiquitination via different protein–protein interaction domains, such as WD40 repeats and leucine rich repeats (LRRs) (see text for details).

complex through its ability to interact independently with Cdc4, Cdc34 and Cdc53 (Kamura et al., 1999; Ohta et al., 1999; Skowyra et al., 1999; Tan, 1999). Cdc4 contains a WD40 repeat domain that specifically binds only to the phosphorylated form of Sic1, thus explaining the requirement for phosphorylation in Sic1 destruction (Feldman et al., 1997; Skowyra et al., 1997). Mutation of several Cdk consensus phosphorylation sites in Sic1 is sufficient to stabilise the protein in vivo (Verma et al., 1997). The SCF^{Cdc4} E3 ubiquitin ligase complex has been reconstituted from recombinant proteins and such complexes are able to ubiquitinate phosphorylated Sic1 in vitro in the presence of Cdc34, E1, ubiquitin and ATP (Feldman et al., 1997; Skowrya et al., 1997). In addition to Sic1, SCF^{Cdc4} targets other important regulatory proteins including the replication protein Cdc6, the Cln-Cdc28 specific inhibitor Far1 and the transcription factor Gcn4 (Kornitzer et al., 1994; Drury et al., 1997; Henchoz et al., 1997). The SCF^{Cdc4} complex is conserved in evolution because a similar complex mediates ubiquitination of the Cdk inhibitor Rum1 and the replication protein Cdc18 in the fission yeast *Schizosaccharomyces pombe* (Kominami and Toda, 1997; Jallepalli et al., 1998; Wolf et al., 1999).

The existence of many F-box containing proteins in sequence databases suggested that the F-box is a protein–protein interaction motif that connects many of these substrate specific adaptor proteins to a common ubiquitination complex, an idea referred to as the 'F-box hypothesis' (Bai et al., 1996). In support of this notion, another F-box protein called Grr1 captures phosphorylated G1 cyclins for ubiquitination by the core SCF machinery (Skowyra et al., 1997, 1999). Similar to Sic1, mutation of Cdk consensus sites in the G1 cyclin Cln2 causes its stabilisation in vivo (Lanker et al., 1996). Grr1 has other targets, including activators of polarised growth called Gic1 and Gic2, and an unknown target in the glucose induction pathway (Li and Johnston, 1997; Jaquenoud et al., 1998). The F-box is a modular domain as different F-box motifs can be functionally interchanged between different proteins across species (Kumar and Paietta, 1998). Many F-box proteins contain specific protein–protein interaction domains, such as WD40 repeats (e.g. in Cdc4, Met30 and β-TrCP) or leucine rich repeats (e.g. in Grr1 and SKP2), which recruit specific substrates to the core complex via Skp1 (Fig. 1c). Recent genetic and biochemical analysis has further elaborated the F-box hypothesis, largely in the yeast system. For example, the F-box protein Met30 antagonises both an activator of *MET* gene transcription and the Cdk inhibitory kinase Swe1 (Kaiser et al., 1998; Patton et al., 1998b).

SCF complexes are evolutionarily conserved. In fact, SKP1 was originally identified in a complex with CyclinA/Cdk2 and the F-box protein SKP2 in human cells (Zhang et al., 1995). Cdc53 homologs in metazoans are termed cullins, after the first identified metazoan homolog, CUL-1 in *Caenorhabditis elegans* (Kipreos et al., 1996). The Cdc53 ortholog, CUL1, assembles into SCF complexes in mammalian cells and is able to complement a *cdc53-2* temperature sensitive *S. cerevisiae* strain (Lisztwan et al., 1998; Lyapina et al., 1998; Yu et al., 1998b). As described below, the function of a number of SCF complexes has recently been determined in a variety of organisms. While it is tempting to generalise that all F-box proteins will participate in E3 ubiquitin ligase complexes, at least one exception should sound a note of caution. That is, although the F-box protein Ctf13 interacts with Skp1 as part of the CBF3 kinetochore complex in yeast, the CBF3 complex apparently does not contain Cdc53, Cdc34 or Rbx1, nor

is there any indication it is a ubiquitin ligase (Connelly and Hieter, 1996; Stemmann and Lechner, 1996; Kaplan et al., 1997).

All the known cell cycle targets of SCF^{Cdc4} and SCF^{Grr1} require Cdk-dependent substrate phosphorylation for recognition by the F-box protein. Thus, Cdk activity coordinates cell cycle progression in large part through the phosphorylation-dependent elimination of inhibitors and activators of various processes in cell division. In addition to regulation of the cell cycle, recent findings suggest that SCF modules have been exploited in the proteolytic control of numerous signalling pathways, as illustrated in several important systems below.

4. Transcriptional regulation by SCF complexes in fungi

Unicellular organisms respond to changes in external nutrient availability by altering the transcription of genes that encode metabolic enzymes and membrane transporters. At least three major metabolic pathways in yeast are regulated by different SCF complexes: glucose induction is mediated by SCF^{Grr1}, methionine repression by SCF^{Met30} and, at least in part, repression of amino acid biosynthesis genes by SCF^{Cdc4}. In each case, the relevant SCF complex appears to target either a transcriptional activator or repressor for degradation. This close interplay between SCF complexes and transcriptional control is also recapitulated in higher eukaryotic regulatory pathways.

4.1. Glucose induction

Glucose is the preferred sugar source of *S. cerevisiae* and as such carbon metabolic pathways are closely attuned to glucose concentration (Johnston, 1999). Glucose regulates its optimal utilisation by both repressing genes required for metabolism of other carbohydrates, such as galactose, and by inducing genes required for glucose catabolism, such as glycolytic enzymes and glucose transporters. The glucose repression pathway is controlled primarily by the Snf1 protein kinase, which phosphorylates a transcriptional repressor called Mig1, thereby sequestering Mig1 in the cytosol (De Vit et al., 1997). Glucose induction on the other hand occurs through Grr1 dependent relief of transcriptional repression, which is mediated by the repressor protein Rgt1 (Ozcan and Johnston, 1995). *grr1* mutants do not express glucose transporter genes such as *HXT1* and are thus severely impaired in their ability to grow on glucose (Flick and Johnston, 1991). Mutations in Grr1, Skp1 or Cdc53, but surprisingly not Cdc34, prevent the induction of glucose transporter genes by glucose (Fig. 2a) (Li and Johnston, 1997). Although an E2 other than Cdc34 may participate with the SCF^{Grr1} complex, the ability of a *cdc34-2* strain to induce a *HXT1* reporter may merely be an allele specific effect (Li and Johnston, 1997). As the Skp1-Grr1 interaction appears to be stimulated by glucose, SCF^{Grr1} activity might be regulated at the level of complex assembly (Li and Johnston, 1997). Despite the powerful genetics of this system, it is has not yet been determined if Rgt1 is the actual target for SCF^{Grr1}, or if another less direct mechanism is at play.

4.2. Methionine repression

Budding yeast have the capacity to utilise inorganic sources of sulphur for the de novo synthesis methionine and cysteine (Thomas and Surdin-Kerjan, 1997). If the availability of methionine and cysteine in the growth medium is limited, the resulting low intracellular concentrations of *S*-adenosylmethionine (AdoMet) induce transcription of biosynthetic genes that assimilate inorganic sulphur into cysteine and methionine. Conversely, high levels of AdoMet repress a large collection of *MET* genes that encode metabolic enzymes and membrane transporters. *MET* gene expression is driven by a transcription complex that contains the proteins Met4, Met28, Cbf1 and, on some promoters, also Met31 and Met32 (Fig.

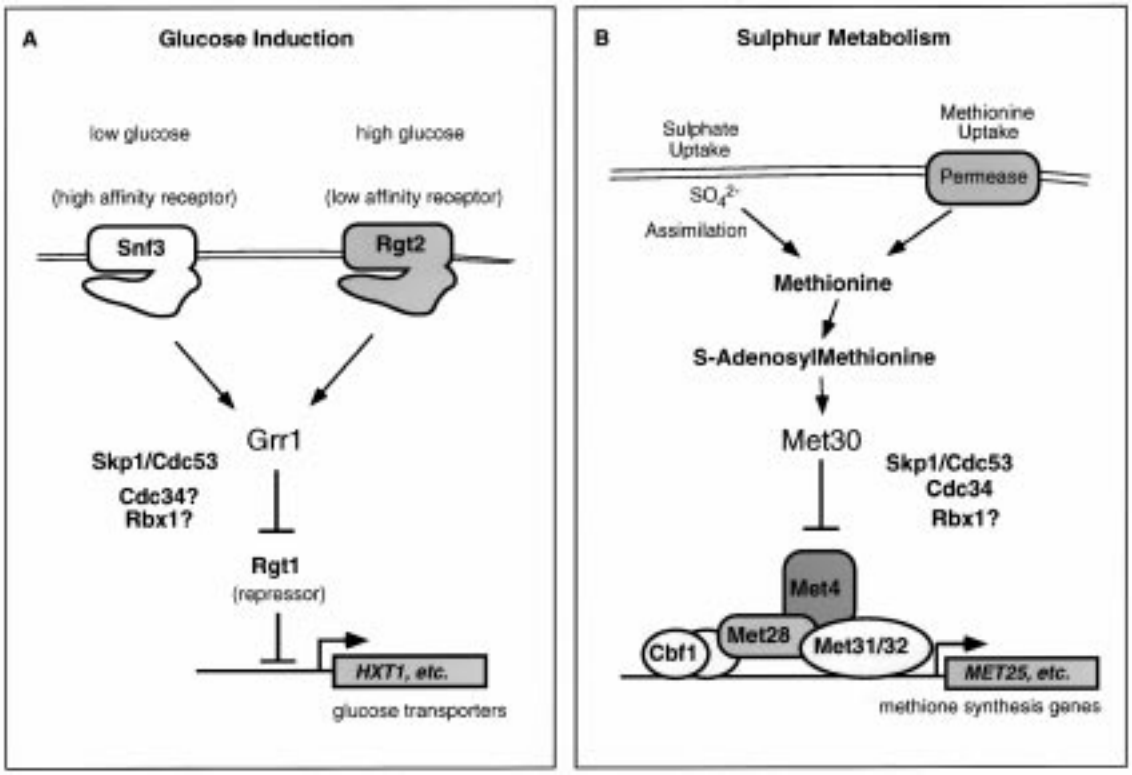

Fig. 2. Transcriptional regulation by F-box proteins in yeast. A) The F-box protein Grr1 is required for transcriptional induction of glucose transporter (*HXT*) genes and represses the Rgt1 transcriptional repressor during growth in glucose medium. Both low (Rgt2) and high affinity (Snf3) glucose receptors function as sensors to transmit the glucose signal to Grr1 by an unknown mechanism. The SCF components Cdc53 and Skp1 are also required for glucose induction, but the roles of Rbx1 and Cdc34 remain to be consolidated. B) The F-box protein Met30 negatively regulates methionine biosynthesis. Under conditions of high intracellular methionine (transported from the extracellular environment by permeases, or synthesised de novo from inorganic sulphur), the metabolite *S*-adenosylmethionine accumulates and activates Met30 by an unknown mechanism. Met30 in conjunction with Skp1, Cdc53 and the E2 enzyme Cdc34 represses the Met activation complex, (composed minimally of Met4, Met28 and Cbf1, but also including Met31 and Met32 on some promoters), which is responsible for stimulating transcription of genes required for methionine biosynthesis (e.g. *MET25*).

2b) (Blaiseau and Thomas, 1998). The F-box protein Met30 was identified in a screen for mutants that constitutively express the *MET25* gene under repressive conditions, and is therefore normally required for repression of many *MET* genes (Fig. 2b) (Thomas et al., 1995). Met30 physically interacts with both Skp1 and, indirectly, Cdc53 through its F-box motif, and contains WD40 repeats presumed to mediate substrate interactions (Fig. 1c) (Patton et al., 1998b). As predicted by the F-box hypothesis, *MET* gene expression is derepressed in temperature sensitive *skp1*, *cdc53* and *cdc34* strains, suggesting that SCF^{Met30} degrades one or more activators of *MET* gene transcription (Patton et al., 1998b). Indeed, Met30 physically interacts with Met4, and moreover, the essential function of Met30 is bypassed by deletion of Met4 (Thomas et al., 1995; D. Thomas, personal communication). However, it is not known if Met4 stability is regulated by methionine levels or furthermore if Met4 is a ubiquitination target of SCF^{Met30}. In addition to its nonessential roles in Swe1 degradation (Kaiser et al., 1998) and *MET* gene repression, Met30 plays an essential function in the G1 to S phase transition, implying the existence of other essential substrates or transcriptional targets of SCF^{Met30} (E. Patton, D. Thomas, personal communication).

Genetic screens in other fungi identified two Met30 homologs, SCON2 in *Neurospora crassa* and SCONB in *Aspergillus nidulans* (Kumar and Paietta, 1995; Natorff et al., 1998). In addition, *sconC*, another *Aspergillus* gene that regulates sulphur metabolism (Natorff et al., 1993), encodes a Skp1 homolog. SCON2 is a negative regulator of sulphur assimilation genes, which are positively controlled by the CYS3 transcriptional activator, a Met4 homolog (Kumar and Paietta, 1998). Thus, the control of sulphur metabolism by SCF complexes appears highly analogous in divergent fungi. Surprisingly, however, point mutations in conserved F-box residues of SCON2 cause constitutive sulphur auxotrophy, completely opposite to the prototrophic phenotype of a *scon-2* null mutant (Kumar and Paietta, 1998). While this result is difficult to reconcile into a model that assumes CYS3 is destabilised by wild-type SCON2, it is possible that such F-box mutants of SCON2 may physically sequester CYS3 and constitutively inhibit its ability to active transcription.

4.3. Amino acid biosynthesis

The expression of some 40 amino acid biosynthetic genes is under the control of Gcn4, a highly unstable protein whose abundance is tightly correlated with its rate of translation. Gcn4 translation is coupled to amino acid availability through the presence of several short open reading frames upstream of the GCN4 initiation codon (Hinnebusch, 1997). The upstream ORFs are preferentially translated under situations of amino acid abundance and so impede translation of the authentic Gcn4 ORF. During starvation, low levels of charged initiator tRNAs result in bypass of initiation at the upstream ORFs, in preference for the downstream Gcn4 ORF. Gcn4 instability is essential in order for the cell to respond rapidly to altered amino acid availability. The SCF^{Cdc4} complex is implicated in Gcn4 degradation because Gcn4 is stabilised in both *cdc4* and *cdc34* strains, although another E2 enzyme, Rad6, also partially mediates Gcn4 instability (Kornitzer et al., 1994; D. Kornitzer, personal communication) As for other known SCF^{Cdc4} substrates, Gcn4 degradation is phosphorylation dependent (Kornitzer et al., 1994). The kinase that phosphorylates Gcn4 to is unknown, but from a

regulatory viewpoint the kinases associated with the transcriptional apparatus, such as Srb10/Srb11 and Kin28/Ccl1, are logical candidates (Hengartner et al., 1998).

Because cellular responses to environmental signals often entail a rapid transcriptional response, many transcription factors are unstable proteins. As described above, each of the F-box proteins of known function in yeast appears to directly regulate transcription, among other processes. It will be interesting to determine if the any of the dozen or so uncharacterised F-box proteins in *S. cerevisiae* also regulate transcriptional programmes. For instance, two novel F-box proteins that are transcriptionally induced during the sporulation programme might participate in transcriptional cascades during meiosis (Chu et al., 1998). Although many details of SCF dependent regulation of transcription factors in yeast still need to be examined, such pathways will undoubtedly serve as important models for transcriptional regulation by SCF complexes in higher species.

5. SCF complexes in plants

5.1. The auxin response

The phytohormone auxin (indole-3-acetic acid) controls cell division and cell elongation in plants during a range of developmental processes including stem elongation, lateral root formation, apical dominance, phototropism and gravitropism (Millner, 1995). In *Arabidopsis*, auxin signalling is blocked by mutations in the *AXR1* (auxin-resistant) gene, which produces stumpy plants that are unable to form lateral roots even when auxin is applied ectopically (Lincoln et al., 1990). Strikingly, AXR1 is similar to the N-terminal half of the E1 ubiquitin activating enzyme (Leyser et al., 1993). AXR1 acts in conjunction with ECR1, which is similar to the E1 C-terminal region (Pozo et al., 1998) to form a heterodimer that is functionally equivalent to a single E1 molecule. However, instead of activating ubiquitin for protein conjugation, AXR1/ECR1 activates a protein called Rub1/Nedd8 that is 50% identical to ubiquitin (Pozo et al., 1998). The precise function of Rub1/Nedd8 conjugation to substrate proteins is unclear, however it is connected to SCF pathways since Cdc53 is covalently modified by Rub1 (Lammer et al., 1998; Liakopoulos et al., 1998). Synthetic lethal interactions between mutations in SCF components and in the Rub1 modification pathway imply that Rub1 is required for optimal SCF function (Lammer et al., 1998). Mammalian cullins appear to be similarly modified by Nedd8 (Osaka et al., 1998).

A compelling connection between the auxin response and SCF complexes arose with the characterisation of another gene required for auxin signalling, *TIR1*, which encodes an F-box protein containing leucine-rich repeats (Ruegger et al., 1998; Pozo and Estelle, 1999). Like *AXR1* mutations, *TIR1* mutations cause defects in hypocotyl elongation and lateral root formation, and furthermore, the two class of mutations synergise with each other. In one model, AXR1 might be required to produce fully active SCF^{TIR1}, which in turn might alleviate repression of the auxin response by elimination of a downstream transcriptional repressor (Fig. 3). Further elaboration of the role of SCF complexes in the auxin response awaits identification of relevant Skp1, Cdc53 and E2 homologs.

5.2. The injury response

Plants have a defence response pathway that may be under control of an SCF complex. Upon tissue injury or infection, oligosaccharides derived from the cell wall in wounded regions elicit the accumulation of jasmonates, which are synthesised from linoleic acid by the action of *FAD* genes (reviewed in Creelman and Mullet, 1997). Together with ethylene produced by insect-damaged tissue, jasmonates trigger the transcription of proteinase inhibitor genes (Botella et al., 1996). Mutations in COI1, an *Arabidopsis* F-box protein with a similar structure to TIR1, attenuates the response to jasmonates, rendering plants highly susceptible to wounds inflicted by chewing insects (Xie et al., 1998). Such mutants also produce pollen that is sterile.

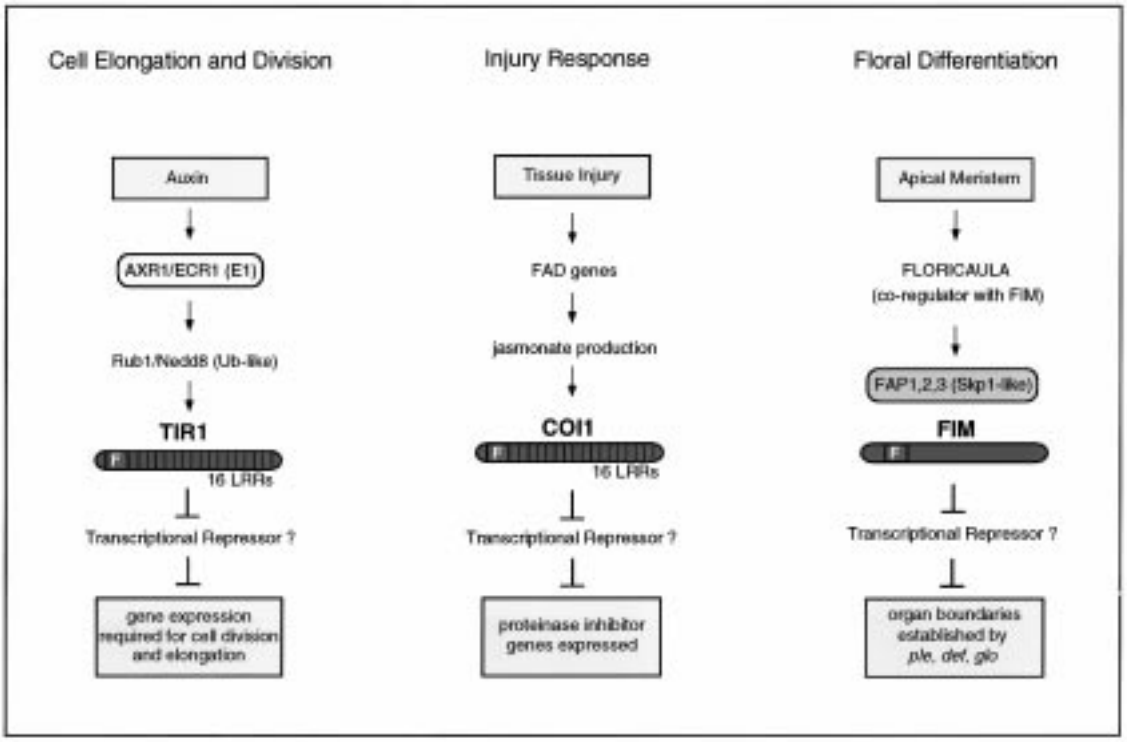

Fig. 3. F-box proteins in plant signal transduction and development. Left panel: in *Arabidopsis*, stimulation of cell elongation and growth is mediated by the hormone auxin. The auxin response requires the E1-like complex AXR1/ECR1, which activates the Rub1/Nedd8 ubiquitin related proteins. The F-box protein TIR1 also functions in this pathway, potentially as a negative regulator of a transcriptional repressor of auxin responsive genes. Middle panel: tissue injury initiates a signalling cascade that activates the FAD genes, which synthesise jasmonates from linoleic acid. Jasmonates induce the transcription of proteinase inhibitor genes by an unknown mechanism that requires the F-box protein COI1, which may act to remove the activity of a downstream transcriptional repressor. Right panel: in snapdragon, floral organ differentiation depends on the general floral induction gene *floricaula* in apical meristems. Together with the *fim* F-box gene, *floricaula* induces transcription of organ identity genes (*ple*, *def*, *glo*), resulting in the proper establishment of morphogenic boundaries during floral organ differentiation. FAP1,2 and 3 are Skp1-like proteins that interact with FIM. A similar pathway based on the FIM ortholog UFO operates in *Arabidopsis*.

By analogy to other SCF pathways, COI1 may mediate the jasmonate response by eliminating a transcriptional repressor, although it still remains to be determined if COI1 functions in the context of an SCF complex (Fig. 3). *COI1* appears to function in a separate signalling pathway from the auxin mediator*TIR1* since *tir1-1* plants are fertile, and conversely, *coi1-1* plants have a normal auxin response (Ruegger et al., 1998; Xie et al., 1998). Thus, in plants, as in yeast, different F-box proteins fulfil nonoverlapping functions.

5.3. Floral differentiation

Flower development requires the differentiation of floral meristems into a pattern of four concentric whorls that specify carpel, stamen, petal and sepal tissue, in an inside to outside order. Floral organ induction is first initiated by the action of the meristem identity genes, *LEAFY* in *Arabidopsis* and *floricaula* in *Antirrhinum* (snapdragon), which activate homeotic genes that specify the distinct floral tissues (Parcy et al., 1998). Mutations in an F-box protein called FIMBRIATA (FIM) in *Antirrhinum* cause defective floral organ development. *fim* mutants display petal/sepal tissue in the second whorl and petal/sepal/carpel tissue in the third whorl, and also display mosaic organs (Simon et al., 1994). FIM may specify organ identity by controlling expression of the MADS box transcription factors *deficiens* (*def*), *globosa* (*glo*) and *plena* (*ple*), which establish morphological boundaries (Ingram et al., 1997) (Fig. 3). In *fim* hypomorphs, *fim* expression is drastically reduced and so decreases and restricts the expression of *def* and *ple* to the centre regions of the floral meristem (Ingram et al., 1997). Since *fim* expression is turned on earlier than *def*, *glo* or *ple*, it might be imagined that FIM-dependent degradation of a transcriptional repressor activates these downstream genes (Ingram et al., 1997). Two orthologs of FIM, unusual floral organs (*UFO*) and *Imp-FIM*, serve analogous functions in *Arabidopsis* and *Impatiens,* respectively (Ingram et al., 1995; Levin and Meyerowitz, 1995; Pouteau et al., 1998). FIM interacts with three Skp1 homologs called FAP1,2,3, (for FIM-associated proteins), that display an overlapping pattern of expression with FIM, suggesting that flower development may be controlled by a family of SCF^{FIM} complexes (Ingram et al., 1997). Roles for either a Cdc53 homologue or an E2 enzyme in floral development have yet to be identified.

6. SCF complexes in animals

6.1. The LIN-12/Notch pathway

The LIN-12/Notch family of transmembrane receptors mediate cell-to-cell communication that induces equivalent cells to adopt different fates, a process known as lateral specification (reviewed in Greenwald, 1998). For example, in *C. elegans*, *lin-12* activity in gonadal precursor cells induces one cell to adopt an anchor cell fate and the other to differentiate into a vulva cell, based on a feedback amplification mechanism that is stochastically initiated within one of the cells. The *Drosophila* and mammalian Notch proteins have a similar role in lateral specification, stimulating differentiation of neuronal tissue and controlling axon guidance. Binding of the Delta/Serrate/LAG-2 (DSL) transmembrane proteins to the Notch receptor in

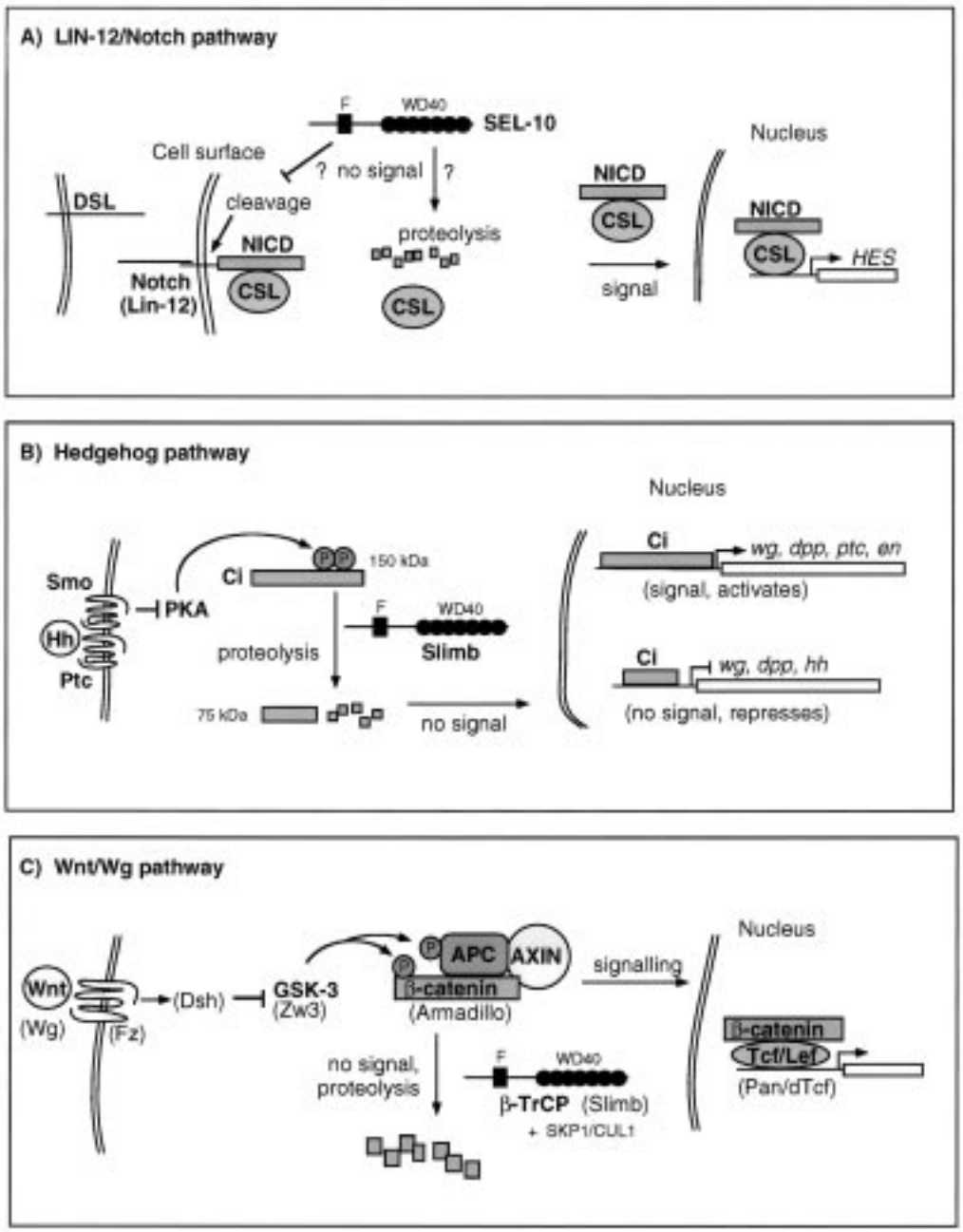

Fig. 4. F-box proteins in animal signal transduction and development. A) The F-box protein SEL-10 represses the Notch/LIN-12 pathway. The Notch receptor (LIN-12 in *C. elegans*) is activated by contact with transmembrane proteins of the Delta/Serrate/LAG-2 (DSL) family in adjacent cells. During signalling, the Notch intracellular domain (NICD) is liberated by a cleavage event at the juxtamembrane region of Notch. In complex with transcription factors of the CBF1/Su(H)/LAG-1 (CSL) family, NICD translocates to the nucleus to activate *Hairy/Enhancer of Split* (*HES*) genes. SEL-10 negatively regulates LIN-12 signalling in *C. elegans* by either inhibiting

(caption continued overleaf)

adjacent cells causes the intracellular domain of Notch to be cleaved. The free Notch intracellular domain (NICD) then translocates to the nucleus, where it activates transcription of *Hairy/Enhancer of Split* (*HES*) genes in conjunction with the CBF1/Supressor of Hairless/LAG-1 (CSL) family of transcription factors (see Fig. 4a) (Chan and Jan, 1998; Struhl and Adachi, 1998). In support of this model, expression of the intracellular domain of LIN-12 phenocopies LIN-12 activation in nematodes (Struhl et al., 1993). Notch signalling can be inhibited by either mutating the cleavage site located in the intracellular juxtamembrane domain of Notch, or by addition of proteasome inhibitors (Kopan et al., 1996; Schroeter et al., 1998).

A clue that SCF complexes may negatively regulate LIN-12/Notch signalling came from the identification of hypomorphic alleles of *sel-10* in a screen for mutations that suppress *lin-12* loss of function in nematodes. SEL-10 is a WD40 repeat and F-box containing protein that physically interacts with the intracellular domain of both LIN-12 and murine Notch4 (Hubbard et al., 1997). Interestingly, *sel-10* loss of function mutants activate Notch signalling even in the absence of ligand, indicating that a small amount of LIN-12/NICD must be liberated and stabilised in the absence of SEL-10. It is not yet known if SEL-10 antagonises Notch signalling by inhibiting the initial cleavage event that liberates the NICD, or whether SEL-10 targets the NCID for degradation (Fig. 4a). Because truncated forms of human NOTCH1/TAN1 cause cell transformation through constitutive activation of the Notch pathway (Capobianco et al., 1997), it is possible that the mammalian counterpart of SEL-10 may function as a tumour suppressor.

6.2. The hedgehog pathway

The hedgehog (Hh) pathway regulates limb development in *Drosophila* by controlling the expression of *wingless* (*wg*) and *decapentaplegic* (*dpp*) in the anterior-posterior axis of wing and leg imaginal discs (reviewed in Hammerschmidt et al., 1997). The F-box protein Slimb (for supernumerary limbs) was identified in a screen for recessive mutations that affect limb development in *Drosophila* (Jiang and Struhl, 1998). *slimb* mutants have duplicated wings and legs, consistent with ectopic Hh signalling. A transcription factor downstream of the Hh

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Fig. 4. *(caption continued)*

release of the NICD, and/or by stimulating proteolysis of the NICD. B) The F-box protein Slimb represses the Hedgehog pathway. In the absence of the Hedgehog (Hh) signal, protein kinase A (PKA) is active and phosphorylates the 150 kDa full-length form of Cubitus interruptus (Ci), thereby targetting the C-terminal region of Ci for cleavage and degradation by a Slimb dependent mechanism. The truncated 75 kDa form of Ci translocates to the nucleus where it represses expression of *wingless* (*wg*), *decapentaplegic* (*dpp*), and *hedgehog* (*hh*). During signalling, the Hh ligand binds to Ptc and removes negative regulation on Smoothened (Smo), which then inhibits PKA and causes the dephosphorylation and stabilisation of Ci. Additional Hh-dependent events are required to convert full-length Ci into an activator of transcription. C) Slimb/β-TrCP inhibits the Wnt/Wg pathway. In the absence of Wnt ligand, GSK-3 phosphorylates β-catenin, which is then recognised and ubiquitinated by the $SCF^{\beta\text{-}TrCP}$ E3 ligase complex. In the presence of Wnt, the Wnt receptor (Frizzled (Fz) in *Drosophila*) indirectly inhibits GSK-3 through Dishevelled (Dsh). β-catenin accumulates and translocates to the nucleus where it activates target genes in a complex with the T-cell factor/lymphoid enhancer factor (Tcf/Lef) family of transcription factors. *Drosophila* homologs are indicated in parenthesis. Pan, Pangolin in *Drosophila*.

pathway called Cubitus interruptus (Ci) has a C-terminal region that is cleaved to yield a 75 kDa form in the absence of Hh signal. The truncated form of Ci lacks a C-terminal cytoplasmic tethering domain, and so translocates to the nucleus where it acts as a transcriptional repressor of the *wg* and *dpp* genes (Aza-Blanc et al., 1997). Cells exposed to Hh accumulate the full-length 150 kDa form of Ci, as do *slimb* mutant cells, indicating that *slimb* is required for Ci proteolysis in the absence of ligand. As loss of cAMP dependent kinase (PKA) activity also causes constitutive activation of the Hh pathway (Chen et al., 1998), it is possible that PKA may phosphorylate Ci, thereby targetting Ci for proteolysis by a SCF^{Slimb} E3 ubiquitin ligase complex (Fig. 4b). Since expression of a stabilised, full-length version of Ci is not sufficient to mediate signalling, Hh must somehow convert full length Ci into a transcriptional activator by a mechanism separate from its prevention of Ci proteolysis (Ohlmeyer and Kalderon, 1998; Methot and Basler, 1999).

6.3. The wingless pathway

In addition to its role in anterior/posterior patterning, Slimb also regulates the wingless (Wg) signalling pathway (Fig.4c), which controls dorsal/ventral patterning in wing and leg imaginal discs (Jiang and Struhl, 1998; Theodosiou et al., 1998; Dierick and Bejsovec, 1999). The Wg ligand stimulates a complex signal transduction pathway that ultimately stabilises a transcription factor called Armadillo (Arm), which activates gene expression in conjunction with the T-cell factor/lymphoid enhancer factor (Tcf/Lef) family of DNA-binding proteins (Fig. 4c) (Behrens et al., 1996; Molenaar et al., 1996). Strikingly, Arm is stabilised in *slimb* mutant cells, suggesting that, in addition to Ci, the Slimb pathway also targets Arm for proteolysis (Jiang and Struhl, 1998). In *Xenopus*, the Arm ortholog β-catenin activates transcriptional events that underlie axis specification, which is similarly antagonised by the Slimb homolog β-TrCP (Marikawa and Elinson, 1998). β-catenin degradation is triggered upon its phosphorylation on a conserved N-terminal residue by the GSK-3/Zw3 kinase. During signalling, the Wg homolog, Wnt-1, represses GSK-3 activity and so stabilises β-catenin, which then translocates to the nucleus to activate target genes (Yost et al., 1996). Elimination of the GSK-3 phosphorylation site on β-catenin or inhibition of GSK-3 activity stabilises and hyperactives β-catenin (Yost et al., 1996).

The regulation of β-catenin degradation has gained considerable interest due to the role of β-catenin in stimulating proliferation and the frequent stabilisation of β-catenin in colon cancer (reviewed in Peifer, 1997). Most cases of hereditary colorectal cancer contain mutations in the adenomatous polyposis coli gene (APC, not to be confused with the Anaphase Promoting Complex, see later), which encodes a β-catenin associated protein (reviewed in Polakis, 1999). A complex between APC and another protein called axin appears to direct the phosphorylation of β-catenin by GSK-3, which in turn triggers β-catenin degradation. Many of the APC mutations identified in colorectal tumours delete several or all of the β-catenin binding sites found in APC (Morin et al., 1997). Conversely, tumours that are wild type for the APC gene contain point mutations and deletions in the β-catenin gene that are predicted to encode a stabilised protein, either by truncation of the N-terminus or mutation of one of three N-terminal serine residues (Morin et al., 1997; Rubinfeld et al., 1997). As anticipated from model system studies, stabilised β-catenin forms an active transcription complex in colon cancer cells

with a member of the Tcf/Lef family (Korinek et al., 1997). Recently, the proto-oncogene *c-MYC*, long known to be overexpressed in colon cancers, has been identified as a target gene for the β-catenin/Tcf transcription complex (He et al., 1998). The activation of cyclin D expression by β-catenin also provides a direct link between Wnt1 signalling and cell proliferation (Tetsu and McCormick, 1999). As predicted by the genetic analysis in *Drosophila*, the mammalian F-box protein β-TrCP specifically binds to phosphorylated β-catenin. Further, over-expression of wild-type β-TrCP destabilises β-catenin, whereas expression of a dominant negative form of β-TrCP stabilises β-catenin (Hart et al., 1999; Latres et al., 1999; Winston et al., 1999). β-TrCP might thus play a tumour suppressor function, although a preliminary survey of colon tumour samples failed to detect any mutations in the β-TrCP gene (Winston et al., 1999).

Interestingly, the control of β-catenin stability may also be linked to familial early-onset Alzheimer's disease, which is associated with mutations in the presenilin-1 gene (reviewed in Selkoe, 1998). In neuronal cells, the presenilin-1 protein normally forms a complex with β-catenin, which appears to stabilise β-catenin. However, in presenilin-1 mutant mice and in tissues from Alzheiner's patients, β-catenin is destabilised, which may lead to increased sensitivity of neuronal cells to apoptosis induced by amyloid-β protein (Zhang et al., 1998). It remains to be determined if β-TrCP function plays any role in neuronal cell survival or the pathology of Alzheimer's disease.

6.4. The NF-κB pathway

The NF-κB transcription factor executes a pattern of gene expression important for inflammatory, immune and stress responses (reviewed in Ghosh et al., 1998; Maniatis, 1999). NF-κB is translated as a 105 kDa precursor protein that is selectively processed to a 50 kDa N-terminal fragment by limited ubiquitin-dependent proteolysis (Palombella et al., 1994; Coux and Goldberg, 1998). Access of NF-κB to the nucleus is regulated by its association with a cytoplasmic anchoring protein, IκBα, which both masks a nuclear localisation signal on NF-κB and inhibits its ability to bind DNA (Arenzana-Seisdedos et al., 1995). Upon activation of the immune response, IkBα is degraded by the ubiquitin system, allowing the NF-κB/RelA complex to translocate to the nucleus (Fig. 5a) (Alkalay et al., 1995). Phosphorylation of IκBα on serine 32 and 36 by a large kinase complex allows recognition of IκBα by an E3 ubiquitin ligase activity (Orian et al., 1995; Cohen et al., 1998; Rothwarf et al., 1998; Zandi et al., 1998). The phosphorylation motif in IκBα is very similar to that in the N-terminus of β-catenin, which hinted that IκBα and β-catenin might be degraded by the same F-box protein (Yaron et al., 1997). The E3 ubiquitin ligase for IκBα has been recently purified and found to contain the human β-TrCP homolog, which binds specifically to phosphorylated, but not unphosphorylated, IκBα (Yaron et al., 1998). Short phosphorylated peptides based on the consensus sequence DpSGXXpS are able to outcompete IκBα and β-catenin for the interaction with β-TrCP (Yaron et al., 1998). As predicted, β-TrCP forms an SCF complex with SKP1 and CUL1, and such complexes act in conjunction with the E2 enzymes Ubc5 or Cdc34 to efficiently ubiquitinate phosphorylated IκBα in vitro (Ohta et al., 1999; Spencer et al., 1999; Tan et al., 1999; Winston et al., 1999). The crucial interaction between phospho-IκBα and β-TrCP may prove to be a useful anti-inflammatory target, although it may be difficult to

selectively inhibit either IκBα or β-catenin degradation without affecting the stability of other as yet identified targets of β-TrCP.

6.5. E2F-1 and cell cycle regulation

Despite the important roles of several SCF complexes in the yeast cell cycle, it has been difficult to discern the much anticipated functions of similar E3 ubiquitin ligases in the mammalian cell cycle. Although cyclin D, cyclin E, and the Cdk inhibitor $p27^{KIP1}$ are all destroyed in a phosphorylation and ubiquitin dependent manner (Clurman et al., 1996; Won and Reed, 1996; Diehl et al., 1997; Vlach et al., 1997), at this point there is no hard evidence to implicate SCF complexes in these degradation pathways. Physical association between cyclin D and SCF complexes has been detected, but the relevance of this interaction remains to rigorously tested (Yu et al., 1998b; Russell et al., 1999). However, new evidence suggests that the E2F-1 transcription factor may be targeted by an SCF complex based on the F-box protein SKP2 (Marti et al., 1999). E2F-1 helps activate the transcription of a number of important genes required for the G1 to S phase transition, including *DHFR*, *cyclin E* and *c-MYC*. Like many other transcription factors, E2F-1 appears to both inhibit and activate gene expression. E2F-1 acts as a repressor when complexed to the Rb tumour suppressor protein in G1 phase, but is converted to an activator once its interaction with Rb is disrupted by phosphorylation of Rb by the G1 cyclin-Cdk kinases (reviewed in Dyson, 1998). Whereas the Rb bound form of E2F-1 is stable, the free form of E2F-1 is actively degraded in a ubiquitin dependent manner (Hateboer et al., 1996; Hofmann et al., 1996). This regulated degradation presumably helps to restrict E2F-1 activity to a window in late G1/S phase. *SKP2* is itself strongly cell cycle regulated, with a peak in S phase shortly after the peak in E2F-1 activity. The nearly coincident timing of *SKP2* expression and the activation of E2F-1 degradation prompted a test for physical interaction between the two proteins, which indeed proved to be the case (Marti et al., 1999). SKP2 appears to bind the N-terminus of E2F-1 as mutants that lack this region are stabilised in vivo and are hypermorphs for entry into S phase. Moreover, transfection of *CUL1* stimulates the formation of ubiquitin conjugates on E2F-1 in vivo. If SKP2 is indeed a limiting factor for E2F-1 degradation, the cell cycle regulation of *SKP2* expression provides the first example of an SCF complex that is regulated by transcriptional control. Despite these strong correlations, proof that E2F-1 is indeed a substrate of the SCF^{Skp2} E3 ligase will require reconstitution of E2F-1 ubiquitination in vitro, and genetic analysis of Skp2 and/or other SCF components in knockout mice. Whether or not recognition of E2F-1 depends on its phosphorylation also remains to be resolved.

7. Viral subversion of an SCF complex

All viruses confiscate aspects of the host cellular machinery, and often this includes manipulation of signalling pathways to create a state favourable for viral replication. With respect to elimination of host functions by rewiring of a degradation pathway, the most famous example is the human papillomavirus virus, which uses its E6 protein to conscript a cellular E3 ubiquitin ligase called E6-AP for the degradation of the tumour suppressor p53

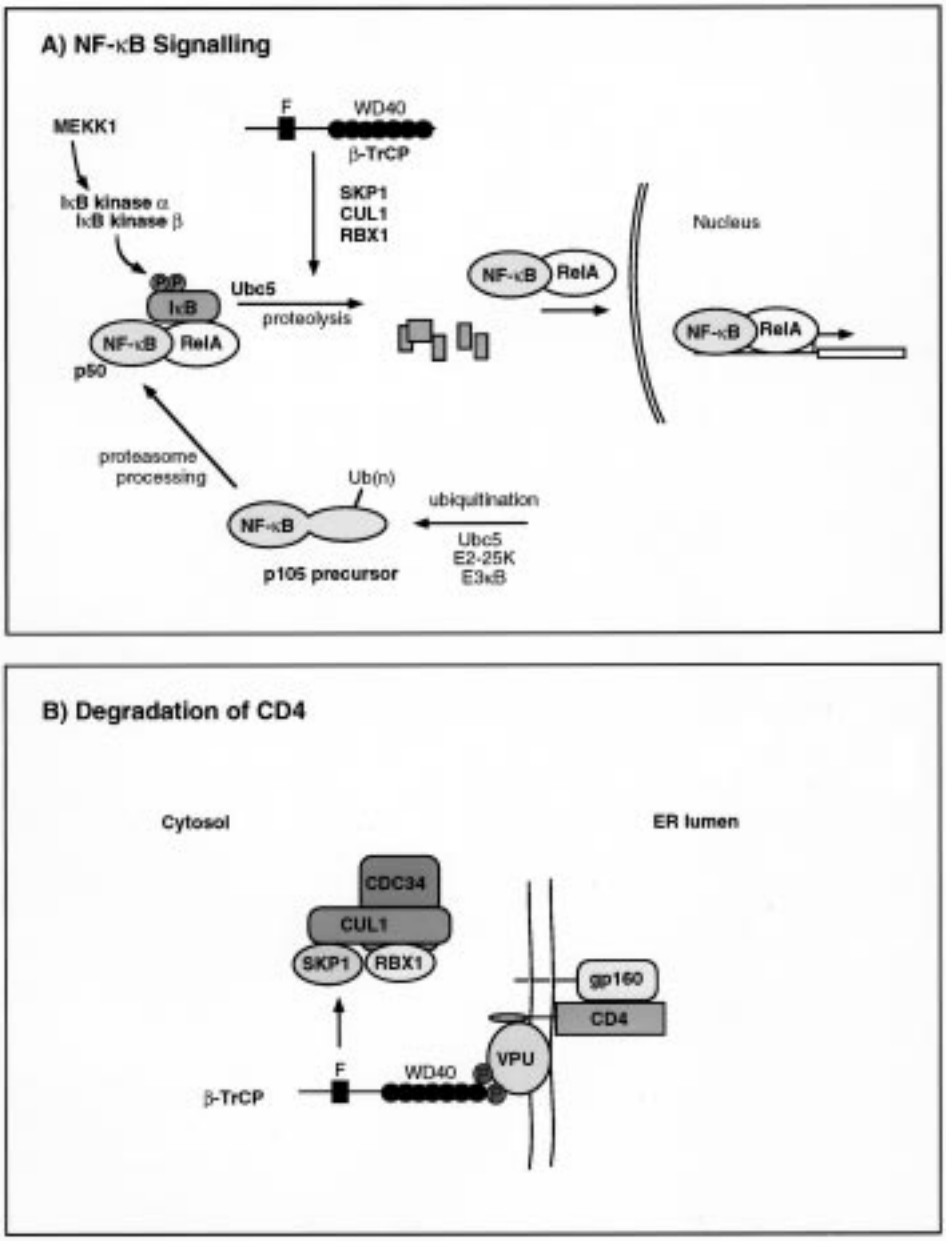

Fig. 5. Proteolysis mediated by β-TrCP. A) Destabilisation of IκBα by the β-TrCP F-box protein. The 105 kDa precursor of NF-κB is processed to a 50 kDa active form by a pathway involving Ubc5 and/or E2-25K, a unique E3 protein ubiquitin ligase (E3κB) and the proteasome. Processed NF-κB is retained in the cytoplasm by anchoring proteins of the IκB family. Signalling (e.g. cytokines, viral stress) activates mitogen activated protein kinase/ERK kinase kinase 1 (MEKK1) which phosphorylates and activates an IκB kinase α/β complex. This kinase complex phosphorylates IκBα, targetting it for degradation by a pathway involving Ubc5 and the F-box protein β-TrCP. Without the IκBα tether, NF-κB is free to translocate to the nucleus where it dimerises with the p65/RelA family of transcription factors to induce expression of target genes. B) HIV utilises β-TrCP to degrade CD4. The HIV envelope precursor gp160 physically interacts with and retains CD4 in the endoplasmic reticulum (ER) of infected cells. Another HIV protein, VPU, is integral to the ER membrane and binds CD4. Two phosphorylation sites on VPU mediate binding to the F-box protein β-TrCP, stimulating degradation of CD4, possibly through an SCF complex containing SKP1 and CUL1, and an associated ubiquitin conjugating activity (CDC34).

(Scheffner et al., 1993). Given the prominence of SCF pathways in cellular regulation, it might be anticipated that F-box proteins or other SCF components will be common targets for viral subversion. In the first known example, HIV exploits an F-box pathway to rid infected T-cells of the CD4 membrane receptor, thereby reducing superinfection and exposure of infected cells to immune surveillance (Margottin et al., 1998). HIV encodes a protein called Vpu that redirects the specificity of the cellular F-box protein β-TrCP to capture and degrade CD4. In infected cells, CD4 is retained in the endoplasmic reticulum by the HIV envelope protein gp160, which facilitates its capture by Vpu. Amazingly, the interaction of the Vpu-CD4 complex with β-TrCP depends on Vpu phosphorylation on two closely spaced serine resides that are embedded in a sequence similar to the β-catenin and IκBα degradation motifs (Fig. 5b). Unlike β-catenin and IκBα, it is not phosphorylated Vpu that is degraded, but the associated CD4 protein. As in β-catenin and IκBα degradation, a version of β-TrCP that lacks the F-box inhibits CD4 degradation in infected cells by titrating CD4 away from the active endogenous SCF complex. A number of F-box proteins and Skp1 homologs are encoded by viral genomes, so other viruses may well exploit cellular SCF pathways as part of their life cycle (Bai et al., 1996).

8. An E3 ligase superfamily

An exciting and unexpected relationship has emerged between SCF complexes and two other E3 ubiquitin ligases, the anaphase promoting complex (APC) and the VHL/ElonginC/ElonginB (VCB) complex (reviewed in Tyers and Willems, 1999). VHL is often mutated in highly vascularised human tumours, such as retinal angiomas, CNS hemangioblastomas and renal cell carcinomas (Kaelin and Maher, 1998). This tumour spectrum may be accounted for by the observation that VHL appears to destabilise hypoxia induced mRNAs, including the angiogenic factor VEGF (Lonergan et al., 1998). VHL is also required for cell cycle exit upon serum withdrawal (Pause et al., 1998). VHL interacts with ElonginB and ElonginC, two proteins that were originally isolated in conjunction with ElonginA as the Elongin SIII complex, which stimulates RNA pol II processivity in vitro (Aso et al., 1996). A member of the cullin family, CUL2, is also part of the VHL complex (Pause et al., 1997; Lonergan et al., 1998). The substantial sequence similarity between ElonginC and Skp1, Elongin B and ubiquitin, and CUL2 and CUL1/Cdc53 insinuated a possible relationship between the VHL and SCF complexes.

This notion has played out in spectacular fashion with the identification of a small ring finger protein of 16 kDa, variously referred to as Rbx1, Roc1 and Hrt1 (referred to here as Rbx1). Rbx1 was initially discovered as part of the VHL tumour suppressor protein complex (Kamura et al., 1999), as a component of purified SCF complexes (Tan et al., 1999), and as a cullin interacting protein (Ohta et al., 1999). In yeast, loss of Rbx1 function causes a similar G1 arrest phenotype as other SCF mutants (Kamura et al., 1999; Ohta et al., 1999). Rbx1 is a versatile protein partner as it independently binds VHL, ElonginB/C, Cdc34, multiple F-box proteins and several cullins, including Cdc53/CUL1 and CUL2 (Kamura et al., 1999; Ohta et al., 1999; Skowyra et al., 1999; Tan, 1999). In particular, Rbx1 enhances the Cdc34–Cdc53 interaction, and in so doing, potently activates the ubiquitination activity of SCF^{Cdc4} towards

Sic1 and SCF^{Grr1} towards Cln2 (Kamura et al., 1999; Skowyra et al., 1999). Similarly, Rbx1 stimulates the activity of the mammalian $SCF^{\beta\text{-}TrCP}$ complex towards phospho-IκBα (Ohta et al., 1999; Tan, 1999).

The occurrence of Rbx1 in both the SCF and VCB complexes strengthened the suspicion that the latter might be an E3 ubiquitin ligase. A further striking similarity between SCF and VCB families is the preponderance of interchangeable adaptor subunits that specifically interact with each type of complex. In addition to VHL and ElonginA, the ElonginB/C heterodimer binds to a large family of proteins, referred to as SOCS-box proteins. The SOCS-box is a sequence motif originally found in suppressors of cytokine signalling (SOCS) proteins, and now identified in more than 20 different proteins (Hilton et al., 1998; Kamura et al., 1998). SOCS box proteins bind to ElonginB/C through a submotif of the SOCS box called the BC box, which is also present in ElonginA and is required for the ElonginA–ElonginB/C interaction (Aso et al., 1996). Interestingly, ElonginA also contains an F-box like sequence, but as yet there is no evidence that it can interact with Skp1. In accord with the host of SOCS-box proteins, the ElonginB/C complex appears to copurify with many different proteins (Kamura et al., 1998). Like F-box proteins, many SOCS-box proteins contain protein–protein interaction motifs such as SH2 domains, WD40 repeats and ankyrin repeats (reviewed in Starr and Hilton, 1999).

The structure of the VHL–ElonginC–ElonginB complex further supports the analogy between SCF and VCB complexes (Stebbins et al., 1999). As predicted from sequence alignments, Skp1 can be closely threaded onto the ElonginC structure, and ElonginB has an overall structure that is very similar to ubiquitin. Based on these observations, it has been suggested that the VHL–ElonginC interface may serve as a template for the corresponding Skp1–F-box interaction surface (Stebbins et al., 1999). VHL has bipartite structure. A C-terminal α helical domain that includes the SOCS box of VHL contacts ElonginC, while an N-terminal β-sheet domain projects away from the ElonginC interface. Intriguingly, superposition of known VHL tumour mutations on the VHL structure reveals two main clusters. The first maps to the ElonginC binding surface, consistent with the disruption of the VHL–ElonginC interaction in such mutants. A second cluster suggests a putative binding surface on the β-sheet domain, which may interact with an unknown partner, possibly a substrate that regulates the stability of hypoxia-induced mRNAs.

In another striking parallel, Rbx1 belongs to the same subclass of ring finger proteins as APC11, a subunit of the APC E3 ubiquitin ligase (Kamura et al., 1999; Ohta et al., 1999; Skowyra et al., 1999). Taken together with the observation that another APC subunit, Apc2, shares overall sequence similarity the cullin family (Yu et al., 1998a; Zachariae et al., 1998b), it seems reasonable to speculate that the APC is distantly related to SCF complexes. Intriguingly, the APC also appears to utilise at least two different substrate recruitment factors, called Cdc20 and Hct1/Cdh1, to target different proteins for degradation at distinct times in mitosis (Schwab et al., 1997; Visintin and Prinz, 1997). Both factors contain a WD40 repeat domain presumed to recruit specific substrates and a common N-terminal region, which might mediate interaction with the APC. The availability of Cdc20 is determined by its transcriptional regulation, whereas the interaction of Cdh1 with the APC is inhibited by Cdk dependent phosphorylation of Cdh1 (Zachariae et al., 1998a; Jaspersen et al., 1999). A meiosis specific Cdc20 homolog called Spo70 (a.k.a. Mct1), identified in a genome wide expression analysis of

meiosis, may direct the APC towards specific targets that are unique to the meiotic cell cycle (Chu et al., 1998). The similar overall architecture of the SCF, VCB and APC complexes is diagrammed in Fig. 6. Whether the Apc11 RING finger protein serves to recruit an E2 enzyme to the APC, and/or whether it nucleates other APC subunits, remains to be determined.

9. Perspective and issues

SCF complexes were first discovered because of their crucial role in the cell cycle-regulated degradation of cyclins, Cdk inhibitors, and replication proteins. The regulatory swath of SCF complexes has widened considerably with recent findings that place SCF complexes in the

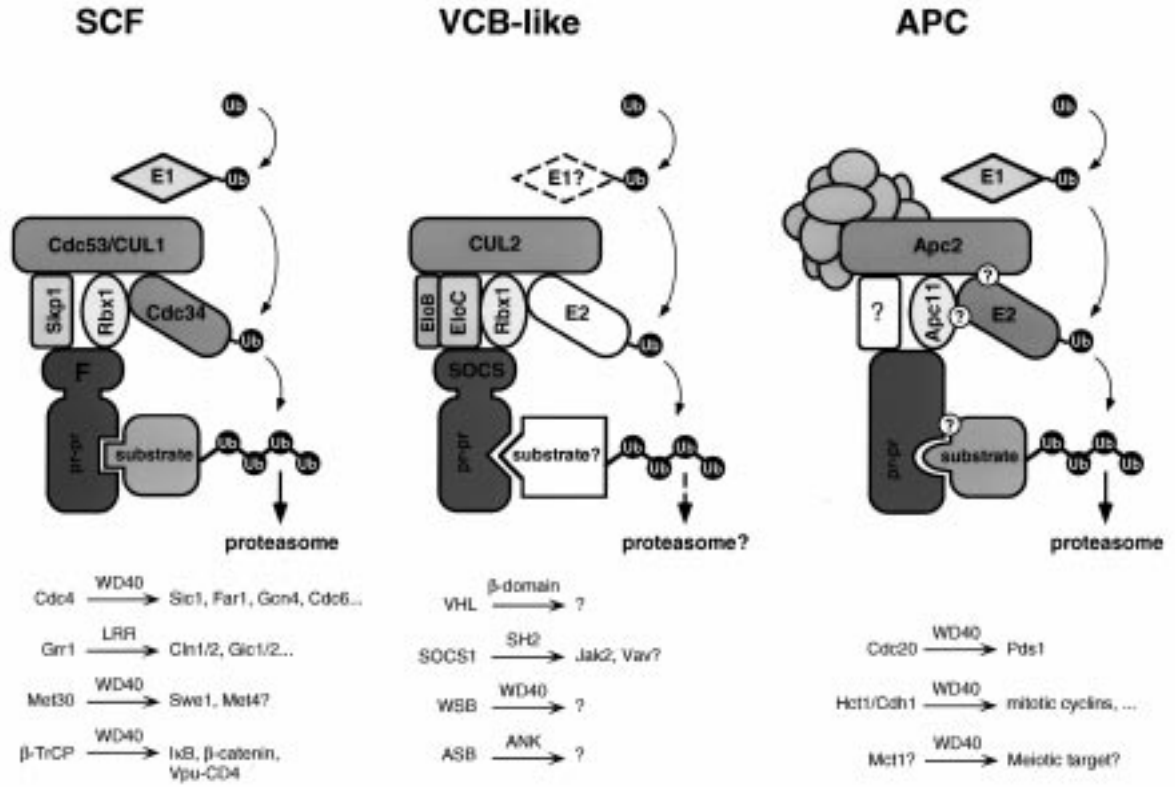

Fig. 6. A superfamily of ubiquitin protein ligases? The SCF, VCB and APC families share a common architecture, and in the case of SCF and VCB, at least one common component (Rbx1). Subunits that share sequence similarity are indicated by the same shade. The SCF and APC complexes are defined ubiquitin ligases, whereas the VCB like complexes are only inferred as such based on their similar overall structure. Each complex uses a set of adaptor proteins that recruit different substrates through specific protein–protein interaction domains. Representative adaptor subunits, their protein–protein interaction domains and their respective substrates are indicated below. Rbx1 and its homolog in the APC, Apc11, may play an integral role in tethering multiple components to each other. Interactions indicated by a question mark are speculative.

midst of important signalling pathways that control development, cell proliferation, immune function and viral pathogenesis. In yeast, plants, nematodes, flies and humans, examples of SCF complexes abound, many of which appear to target transcription factors for degradation. As originally predicted by the F-box hypothesis (Bai et al., 1996), the potential interaction of many different F-box proteins with the Skp1/Rbx1/Cdc53/Cullin core complex might allow a huge number of specific substrates to be targeted for degradation. In the first comprehensive metazoan example, more than 100 putative F-box proteins, 10 Skp1 homologs and 5 Cdc53 homologs can be identified in the recently completed *C. elegans* genome (The *C. elegans* Sequencing Consortium, 1998).

Given the existence of literally hundreds of F-box proteins in sequence databases, an outstanding issue is the identity of the relevant substrates for the phalanx of presumed SCF complexes. This problem encompasses not only the putative F-box proteins of unknown function revealed by genome sequencing projects but also well understood F-box proteins, such as Cdc4, Grr1, Met30 and β-TrCP, which may regulate yet other pathways in addition to those delineated thus far. The developmental regulation of several new F-box proteins suggests specific roles in organogenesis in plants, in the mitotic cell cycle in mammalian cells and in the meiotic programme of yeast. However, assigning function to unknown F-box proteins is not trivial, even in genetically tractable organisms such as yeast. For example, although several new F-box proteins in yeast clearly form SCF complexes, genetic analysis has failed to identify any obvious role for these complexes (Willems et al., 1999). On the other hand, a complementary dilemma exists for several important substrates that are destroyed in a phosphorylation and ubiquitin dependent manner, including cyclin D, cyclin E and $p27^{KIP}$. While expectations are high that these cell cycle regulators will be targeted by SCF complexes, there is no concrete data to support this prediction.

Identification of the relevant kinases and their attendant regulatory pathways is obviously essential for understanding how the degradation of many SCF substrates is programmed. Although phosphorylation appears to be the most common mechanism that dictates substrate recognition, other mechanisms also probably confer selectivity, such as regulated F-box protein expression, and regulated assembly of SCF complexes, as noted above. Interestingly, at least some F-box proteins are themselves rather unstable proteins that, somewhat ironically, are degraded in an SCF dependent manner (Zhou and Howley, 1998; Kaplan et al., 1997). Finally, SCF complexes may also undergo regulatory modifications, such as the modification of Cdc53 and other cullins by the ubiquitin related protein Rub1/Nedd8. Such postranslational modifications might alter the localisation, assembly or activity of SCF complexes, as suggested by genetic analysis of the Rub1/Nedd8 pathway in yeast and plants.

The similar architecture of the SCF, VCB and APC families raises many intriguing possibilities. If VCB complexes do indeed turn out to be E3 ubiquitin protein ligases, what are the substrates of the numerous SOCS-box proteins? As SOCS-box proteins are implicated in attenuation of cytokine signalling pathways, the proteins that transmit such signals are obvious candidate targets. For instance, the SH2 containing protein SOCS1 interacts both with the kinase Jak2 and with the exchange factor Vav (De Sepulveda et al., 1999), either of which might be substrates for a VCB-like E3 ubiquitin ligase. Will the access of such substrates to their cognate VCB complex be regulated in some manner, perhaps by phosphorylation, or perhaps by signal transmission? With regard to the APC, are there other adaptor proteins, and if so, will

they function in mitotic regulation exclusively, or might they also regulate other processes as well? Lastly, function has yet to be ascribed to the remainder of the cullin and Skp1 family members, some of which might define yet other classes of SCF-like E3 ubiquitin ligases.

With respect to the enzymatic mechanism of the SCF E3 ubiquitin ligases, much remains to be settled. How does the presence of Rbx1 in the fully assembled SCF complex stimulate the activity of Cdc34 (Skowyra et al., 1999)? Might Rbx1 act as a catalytic intermediate in the transfer of ubiquitin? While preliminary mutagenesis studies are consistent with this idea (Ohta et al., 1999), Rbx1 contains many cysteine residues that are essential for Zn^{2+} binding by the RING finger domain, and so it is difficult to rule out structural perturbations caused by loss of one or more cysteines. Do all SCF complexes rely on Cdc34 as a ubiquitin donor, or do other E2 enzymes participate in vivo, as suggested by the ability of Ubc5 to mediate IκBα ubiquitination in vitro (Ohta et al., 1999)? The association of multiple E2 enzymes into SCF complexes may increase the combinatorial potential of SCF pathways. What is the actual catalytic mechanism — does a single SCF complex completely ubiquitinate a substrate before release occurs or is the reaction more complex, perhaps involving substrate transfer between two or more SCF complexes? Recent evidence in *S. pombe* suggests that the $SCF^{Pop1/2}$ complex, which targets the Cdk inhibitor Rum1 for degradation, may indeed require dimerisation of the SCF complex for activity in vivo (Wolf et al., 1999). Finally, will the structure of the Skp1–F-box interface mimic the ElonginC–VHL interface, or are more surprises in store?

The structural basis for phosphorylation dependent substrate recognition is a central issue, particularly if effective inhibitors of specific degradation pathways are to be designed for therapeutic uses. At least in some instances, phosphorylation dependent recognition by an F-box protein is relatively straightforward, as in the case of IκBα or β-catenin, which probably bind β-TrCP through a simple phosphate dependent ionic interaction. In other cases, the mechanism of binding may be much more complex. For instance, the many known SCF^{Cdc4} substrates do not appear to share any common sequence motif other than Cdk phosphorylation sites. Moreover, some Cdc4 substrates such as Sic1 must be phosphorylated on multiple sites to drive recognition (Verma et al., 1997), while others, such as Far1, appear to require phosphorylation on only a single site (Henchoz et al., 1997). In these instances, whether binding depends on direct ionic interactions or phosphate driven exposure of a cryptic binding epitope, or both, is unclear. It is also perplexing that apparently similar polyphosphorylated substrates, such as Sic1 and Cln2, are recognised by completely different protein–protein interaction domains, the WD40 repeats of Cdc4 and the leucine rich repeats of Grr1, respectively. Regardless of the precise mechanisms, it is evident that phosphorylation has evolved as a primary means to decide precisely when important proteins are selected for degradation by the ubiquitination machinery. The vast regulatory network based on the SCF, VCB and APC E3 ubiquitin ligase families now seems certain to underlie much of the programmed proteolysis in the cell.

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