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Review

The utility F-box for protein destruction

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Abstract. A signature feature of all living organisms is their utilization of proteins to construct molecular machineries that undertake the complex network of cellular activities. The abundance of a protein element is temporally and spatially regulated in two opposing aspects: *de novo* synthesis to manufacture the required amount of the protein, and destruction of the protein when it is in excess or no longer needed. One major route of protein destruction is coordinated by a set of conserved molecules, the F-box proteins, which promote ubiquitination in the ubiquitin-proteasome pathway. Here we discuss the functions of F-box proteins in several cellular scenarios including cell cycle progression, synapse formation, plant hormone responses, and the circadian clock. We particularly emphasize the mechanisms whereby F-box proteins recruit specific substrates and regulate their abundance in the context of SCF E3 ligases. For some exceptions, we also review how F-box proteins function through non-SCF mechanisms.

Keywords. F-box, SCF, 26S proteasome, protein degradation, ubiquitination.

Introduction

The F-box hypothesis

Traced back to the 1990, a region of homology was initially observed in proteins containing β-transducin repeats like Cdc4, Met30, and β-TrCP [1]. This homologous sequence was not characterized until the discovery of a novel gene, *Skp1p*, via a suppressor screen for *cdc4–1* [2]. Two-hybrid searches conducted for Cyclin F-binding proteins have also identified the human counterpart Skp1 [3, 4]. *Skp1* genes are crucial for cell cycle regulation and influential in determining the stability of certain proteins. Alignments of Skp1p-interacting protein sequences by Elledge and colleagues have revealed a degenerate 40-amino-acid sequence required for binding to Skp1p [2]. It was not until then that a name, 'F-box' was coined for this motif, subsequent to which, the 'F-box hypothesis' arose.

The term F-box was named after Cyclin F., the first defined F-box protein (FBP) [2]. Soon after, proteins carrying the F-box motif were found to be evolutionarily conserved in various species. To date, numbers of identified FBPs range from ~11 in *Saccharomyces cerevisiae*, ~30 in *Drosophila melanogaster*, to ~600 in *Arabidopsis thaliana*. Previous characterizations have revealed that FBPs possess diverse functions and are often implicated in the ubiquitin-proteasome system which mediates protein destruction by covalently attaching ubiquitin moieties (Ub) onto a substrate [5–8]. In this system, the E1 enzyme activates the Ub moiety whereas the E2 enzyme is responsible for the conjugation. FBP is a component of an E3 ligase, the

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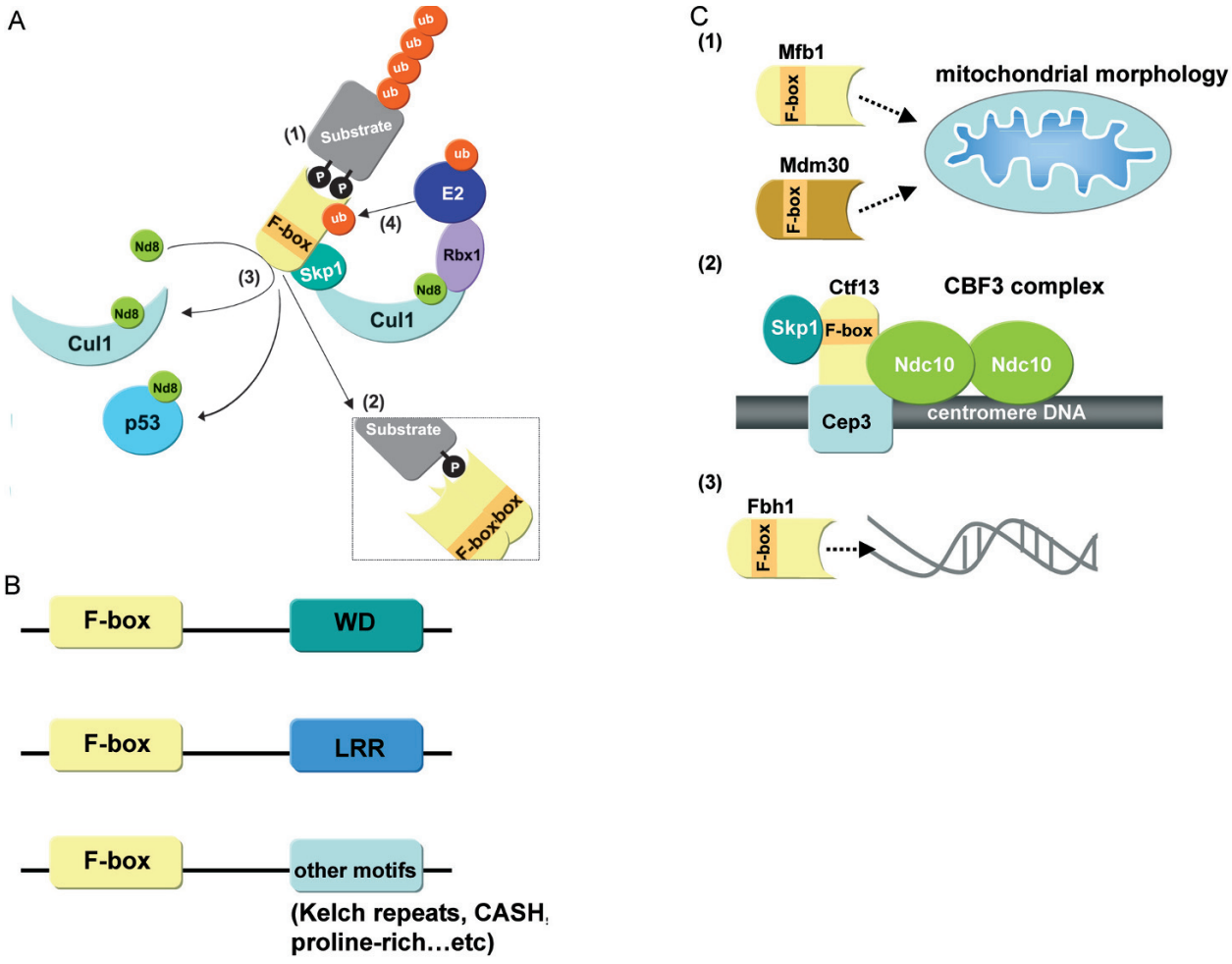

Figure 1. The FBP is a component of the SCF complex and possesses diverse functions. (*A*) The SCF complex is an E3 ligase that mediates the ubiquitin (Ub) transfer from the E2 conjugating enzyme to the targeted substrate. It is composed of the scaffold protein Cul1, which interacts with Skp1 at the N terminus and the RING domain protein Rbx1/Roc1/Hrt1 at the C terminus. Cul1 is conjugated with the Ub-like moiety Nedd8 (Nd8) important for its E3 ligase activity. Rbx1 recruits the E2 enzyme carrying the Ub, and Skp1 interacts with the FBP (yellow; the F-box domain is highlighted in orange). The FBP possesses multiple functions (1) it binds to phosporylated substrates and targets their ubiquitination; (2) it is capable of forming dimers; (3) it is involved in the neddylation of Cul1 and p53; (4) it itself is a ubiquitination target. (*B*) Typical FBP protein structures. FBPs carry the F-box motif at the N terminus, and different protein-protein interaction motifs like WD repeats or LRRs at the C terminus. (*C*) The FBP works independently of the SCF complex. (1) FBP Mfb1 and Mdm30 regulate mitochondrial morphology. (2) FBP Ctf13 and Skp1 are parts of the yeast CBF3 complex for centromere binding. (3) FBP Fbh1 is a helicase that unwinds DNA.

SCF complex (Skp_Cul1_F-box), which mediates the Ub transfer from E2 to a targeted substrate. Within SCF complexes, FBP binds to Skp1 which interacts with the scaffold protein Cul1 at its N terminus. The C terminus of Cul1 interacts with a RING domain protein Rbx1/Roc1/Hrt1 which binds to the Ub-loaded E2 (Fig. 1A). An F-box hypothesis was then established and refers to the action of FBPs that serve as the variable adaptors to recruit specific protein substrates to SCF complexes [9, 10]. In addition to the shared F-box domains at the N terminus, FBPs often carry other domains such as WD40 repeats (WD40) or leucine-rich repeats (LRRs) at their C terminus for substrate recognition. WD40 repeats display β propeller structures, and LRR repeats are arc-shaped α-β repeats that mediate protein-protein interaction [11–13]. Some other domains such as Kelch repeats, CASH (carbohydrate-interacting), and proline-rich domains are also present at the C terminus of FBPs (Fig. 1B) [14].

Structures of SCF^{FBP} complexes

Crystal structures of SCF complexes have been solved to understand the mechanism of Ub transfer from E2 to a targeted substrate [14–16]. In these structures, components of the SCF complex are organized into a single rigid C-shaped macromolecular structure spanning a distance of ~59 Å from end to end. At one end

of the structure is the E2 enzyme carrying the Ub, while the substrate-recognition domain of FBP is positioned on the other end. One of the current models suggests that the 59-Å distance is critical for the Ub transfer. To maintain the rigidity of this interface, Skp1, Cul1, and FBP need to be correctly oriented in space to allow the transfer reaction to proceed. The FBP linker region between the F-box and the substrate-recognition domain is also determinative. This flexible linker segment is suggested to affect the coupling between the two domains and thus alter the rigidity of the whole enzymatic surface [15]. Moreover, the interface between Skp1 and the F-box domain is equally important in mediating the Ub transfer. The F-box domain is envisioned as a trihelical structure, and several key residues within the domain are involved in the formation of a hydrophobic surface for binding to Skp1 [14]. *In toto*, these lines of evidence suggest that the spatial position and the structural function of FBPs are particularly crucial for the organization and the activity of SCF enzymatic complexes.

Phosphorylation is required for FBP target recognition

FBPs in SCF complexes are known to provide the specificity when deciding on which substrate to degrade. In the majority of the model organisms, substrate phosphorylation is one common prerequisite for FBP target recognition (Fig. 1A). It is crucial that one or more residues of the substrate phosphodegron, a sequence with which FBPs specifically interact, are phosphorylated prior to the FBP-substrate interaction. Analysis of a well-characterized mammalian FBP, β-TrCP, indicates that most of its canonical substrates contain a DpSGΦXpS motif in which Φ is a hydrophobic amino acid and pS is a phosphoserine. The two serine residues in the motif need to be phosphorylated sequentially before binding to β-TrCP. For example, IκBα carries a phosphodegron $EDpS_{32}GLDpS_{36}$ and the IκB kinase complex is required for the serine phosphorylation before β-TrCP recognition [17–19]. Another well-known mammalian FBP, Fbw7/hCdc4, recognizes the phosphodegron (CPD) of the consensus LLpTPPQSG deduced from Cyclin E [15, 20–22]. Within the Cyclin E CPD sequence, Thr-380 is the crucial phospho residue with an immediately adjacent proline residue [21]. Some Fbw7 substrates also carry a phosphorylated serine at the +4 position after the threonine. Sequential phosphorylation events in some cases are important for substrate binding. Many substrates include more than one phosphodegron, as in the case of Sic1p, the substrate of the yeast Cdc4p, the significance of which will be discussed in more detail below.

FBP dimerization

Due to the presence of multiple phosphodegrons in their substrates, some FBPs are proposed to act as dimers (Fig. 1A). Mammalian FBPs β-TrCP1 and β-TrCP2 (also known as HOS) have been shown to form homo- or heterodimers that regulate IκBα [23]. The dimerization event requires the D-domain (a region just upstream of the F-box), and only homodimers of each participate in the ubiquitination and degradation of IκBα. On the other hand, Fbw7 contains three differentially spliced isoforms: α, β, and γ [21, 24–26]. The Fbw7α isoform resides mainly in the nucleoplasm, while the Fbw7β isoform is cytoplasmic. The γ isoform of Fbw7, however, is localized exclusively in the nucleolus. Overexpression studies have shown that Fbw7 isoforms form homo- or heterodimers [27]. Interaction assays have also narrowed the Fbw7 dimerization domain down to the region just upstream of the F-box domain [27]. Since three isoforms were found in different subcellular compartments, it is tempting to speculate that heterodimer formation alters the original localization of Fbw7 isoforms and regulate each others' activity. Moreover, dimerization event has also been observed for the mammalian FBP Skp2, the fission yeast FBPs Pop1p, and Pop2p [28–31]. Altogether, these observations suggest that FBP dimerization is a general phenomenon and might be conditionally required for its target recognition.

FBPs regulate neddylation of Cul1 and p53

An intriguing aspect of FBPs centers on how they regulate other types of post-translational modifications. Neddylation, in particular, was found to be regulated by the FBPs (Fig. 1A). Similar to ubiquitination, neddylation is a process that conjugates the Ub-like moiety Nedd8 to cullins via Nedd8-specific enzymes E1, E2, and E3. It is commonly accepted that neddylation of Cul1 improves the E3 ligase activity and that the Nedd8 moiety is detached from Cul1 by the action of the COP9/signalosome (CSN) complex [32–34]. By providing an extra amount of the FBP Skp2, Skp1, and the substrate p27, Cul1 is increasingly neddylated in human cells, suggesting that the presence of the FBP and its substrate alters Cul1 neddylation levels [35]. Furthermore, the inhibitory factor, CAND1, is required to bind to the non-neddylated Cul1 to prevent its neddylation [36, 37]. Addition of Skp2 and substrate overpowers the effects of CAND1 and prevents the deneddylation action performed by CSN [35].

FBPs also regulate the neddylation of a non-cullin substrate p53. FBXO11, an FBP that works in concert

with the SCF complex, promotes the neddylation of p53 at two lysines within its nuclear localization signal [38]. Interestingly, FBXO11 does not promote p53 ubiquitination *in vivo* and the stability of p53 is not affected. It is hypothesized that FBXO11 regulates p53 localization, thus affecting its transcription activity and suppressing the function of p53.

FBPs as ubiquitination targets

As described above, FBPs are positioned in the enzymatic interface to bridge substrates for accepting Ub; this structural placement also allows FBPs themselves to become ubiquitination targets (Fig. 1A). For example the FBP Cdc4p within the *S. cerevisiae* SCF^{Cdc4p} complexes was found to be short-lived and subjected to autoubiquitination [39, 40]. The presence of a motif, termed the R-motif (for 'reduced abundance'), influences the half-life of Cdc4p [41]. These observations raise the possibility that, as the stabilities of FBPs are dynamically regulated, they are probably interchangeable within individual SCF complexes. Once an FBP is ubiquitinated and degraded, SCF complexes are available for a new FBP molecule of the same or different kind for further action. Similarly, the mammalian FBP Skp2 regulates the G1-S transition during cell cycle progression and is ubiquitinated through an autocatalytic mechanism mediated by Cul1-containing SCF complexes. The suppression of Skp2 protein levels is crucial in the quiescent fibroblasts during the G0-G1 phases of the cell cycle [42].

Experiments have also shown that increased substrate levels lead to the stabilization of the corresponding FBP. For example the ubiquitination and degradation of FBP β-TrCP2 were found to be hindered when protein levels of the phosphorylated substrate IκBα increase [43]. This result suggests that substrate availability plays a role in protecting the FBP from autoubiquitination. Presumably, when the substrate levels are low, the FBP is no longer physically shielded from the interaction with the substrate, and becomes available for catalytic destruction by the SCF complex. Furthermore, in addition to promoting the Cul1 neddylation, the FBP is destabilized in CSN-deficient cells [34, 44, 45]. This evidence suggests that the CSN complex protects the FBP from autoubiquitination and that CSN-protected FBPs might then be recycled and available to generate another set of pools of activated SCF complexes [34, 45].

FBPs possess SCF-independent functions

Some FBPs also act independently in an SCF-free context, albeit associations betwee Skp1 and FBP are still commonly seen. Growing evidence has indicated that FBP associates with Skp1 but performs SCF-independent functions [7, 46]. For example, in budding yeast, the FBP Mfb1 and Mdm30 regulate mitochondrial morphology by controlling mitochondria fusion and tubule formation (Fig. 1C) [47]. The yeast CBF3 kinetochore-binding complex containing the FBP Ctf13 and Skp1 is a fundamental centromere-binding structure (Fig. 1C) [48]. Moreover, the FBP Fbh1 was the first one found to possess intrinsic enzymatic activity and is a DNA-dependent ATPase with a DNA-unwinding helicase activity (Fig. 1C) [49, 50]. Studies of the fission yeast FBPs also indicate that they react to encountered stressors such as oxidative or osmotic stresses [51, 52]. Thus, FBPs, as intrinsically unstable proteins in many cases, exhibit diverse functions in mediating environmental stress responses and cell cycle regulation, in addition to serving as the substrate adaptors for the core SCF E3 ligases.

It is known that ubiquitination of transcription factors is tightly coupled to gene transcription [53, 54]. For example, the turnover of the yeast transcription factor Gal4 is mediated by the FBPs in two ways. Under non-inducing conditions, Gal4 is destroyed via the FBP Grr1. Grr1-mediated degradation of Gal4 has resulted in the inactivation of Gal4-dependent gene transcription. On the other hand, once Gal4 is activated in galactose media, the FBP Dsg1/Mdm30 is required to regulate Gal4 protein stability. When Dsg1/Mdm30 is absent, Gal4 target genes are efficiently transcribed. However, Dsg1/Mdm30 was found to be essential for the production of functional messenger RNAs during Gal4 transcription, indicating that Dsg1/Mdm30 is involved in the cotranscriptional mRNA-processing step instead [55].

In this review, we focus on FBP functions in the cell cycle and genomic instability, synapse formation, *Drosophila* tissue development, plant hormone responses, plant organ formation, light signaling and circadian rhythm, viral and bacterial infection, and glycoprotein quality control (Table 1). In an attempt to cover the research as extensively as possible, we aim to provide a thorough overview on how FBPs function in various cellular settings, and apologize if some of the work is not present due to the length limitation of the article.

FBPs in cell cycle and genomic instability

Owing to their functional diversity, it is not surprising that FBPs regulate the cell cycle, an orderly sequence of events including DNA synthesis (S phase) and mitosis (M phase). Distinct cycling phases enable cells to divide correctly and maintain genome integrity, whereas disruptions of normal cycling by inappropriate timing in phase entry are detrimental. Cell cycle

Table 1. FBPs discussed in this review.

Name/species	Substrate	Special domains	Function
Fbw7(hSEL-10, hCdc4)/*Hs*	c-Myc, Cyclin E	WD40	cell cycle and viral infection
Cdc4p/*Sc*	Sic1p	WD40	cell cycle
Ago/*Dm*	Cyclin E., dMyc	WD40	cell cycle
SEL-10/*Ce*	?	WD40	synapse elimination
β-TrCP/*Hs*	Emi1, Wee1A, Cdc25A	WD40	cell cycle and viral infection
Slimb/*Dm*	PER	WD40	animal circadian clock
LIN-23/*Ce*	BAR-1(β-catenin) and unknown target	WD40	WNT signaling, glutamate receptor stability, and axon outgrowth
Skp2/*Hs*	p27, c-Myc, Cyclin E	LRR	cell cycle
TIR1/*At*	AUX/IAA	LRR	auxin response
ABF5/*At*	?	LRR	synthetic auxin
EBF1 and 2/*At*	EIN3 and EIL	LRR	ethylene response
UFO/*At*	?	?	floral development
FIM/*Antirrhinum*	?	?	floral development
MAX2/*At*	?	LRR	shoot branching
ARABIDILLO-1 and 2/*At*	?	arm-repeats	lateral root formation
CEG/*At*	?	?	lateral root formation
VFB1-4/*At*	?	LRR	lateral root formation
AhSLF-S2/ *Antirrhinum*	S-RNase	?	self-imcompatibility
EID1/*At*	?	leucine zipper	PhyA signaling
AFR/*At*	?	Kelch repeats	PhyA signaling
ZTL/*At*	TOC1, PRR5	LOV, Kelch repeats	plant circadian clock
FKF1/*At*	CDF1	LOV, Kelch repeats	plant circadian clock
LKP2/*At*	?	LOV, Kelch repeats	plant circadian clock
JET/*Dm*	TIM	LRR	animal circadian clock
FBXL3/*Hs*	CRY	LRR	Animal circadian clock
P0/Polerovirus	AGO1	?	viral infection
VirF/*Agrobacterium*	VIP1	?	bacterial infection
Fbs1(Fbx2, OCP1)/ mouse	pre-integrin β1, NMDA subunit NR1	SBD	glycoprotein quality control

Hs, *Homo sapiens*; *Sc*, *Saccharomyces cerevisiae*; *Dm*, *Drosophila melanogaster*; *At*, *Arabidopsis thaliana.*

defects such as abnormal cell divisions confer genomic instability and might render cells oncogenic. Therefore, organisms devise complex mechanisms to regulate cell cycle progression, entry, and exit. One classical regulation is provided by the degradations of cell cycle components such as cyclins and cyclin-dependent kinases (Cdks), and we discuss in particular the FBP-mediated degradation mechanism (an overview is illustrated in Fig. 2A).

Skp2 regulates Cdk inhibitors during the G1-S transition

Skp2 binds and mediates the degradation of p27, its first identified function in the cell cycle [56–59]. p27 is an inhibitor of Cdk2 (CKI) and forms a ternary complex with Cyclin E-Cdk2. Further results showed that Skp2 targets p27 with the Thr-187 residue phosphorylated by Cyclin E-Cdk2 complexes [60, 61], suggesting that levels of p27 are regulated by a feedback regulatory loop. Thus, the ubiquitination of p27 by Skp2 frees and activates Cyclin E-Cdk2 complexes, thereby allowing cells to enter the S

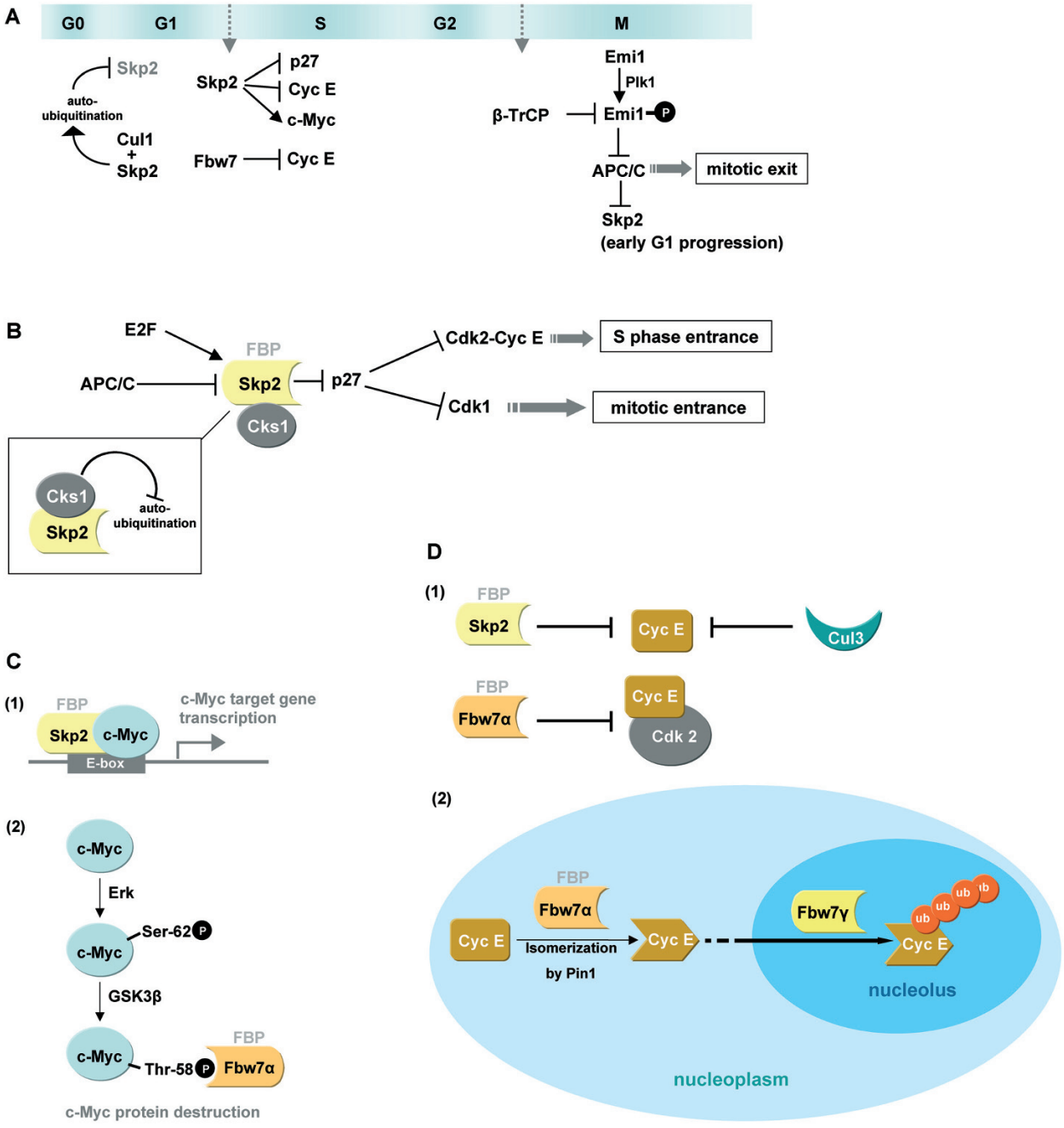

Figure 2. FBPs in the cell cycle. (*A*) An overview on how FBPs regulate the cell cycle. During G0 and G1 phases, the FBP Skp2 undergoes autoubiquitination by Cul1-containing SCF complexes and its protein levels are suppressed. During the G1 to S transition, Skp2 mediates the degradation of p27 and Cyclin E (Cyc E) whereas Fbw7 controls the protein levels of Cyc E. For c-Myc, Skp2 serves as a transcriptional activator to induce its activity. During the G2 to M progression, the APC/C complex inhibitor, Emi1, is phosphorylated by the kinase Plk1 and degraded in a β-TrCP-dependent manner. β-TrCP-mediated Emi1 ubiquitination regulates the activity of APC/C complexes and mitotic entry. The APC/C complex also regulates the protein levels of Skp2 in early G1 progression. (*B*) FBP Skp2 mediates the degradation of p27. This action is important for both S phase and mitotic entrance since p27 is important for the activity of the Cdk2-Cyc E complex and Cdk1, respectively. The Skp2 transcript level is regulated by E2F transcription factors, and the APC/C complex is known to suppress the activity of Skp2. Skp2 autoubiquitination is prohibited when Cks1 interacts with Skp2. However, Cks1 promotes Skp2-dependent p27 ubiquitination. (*C*) The degradation of c-Myc is mediated by two FBPs: Skp2 and Fbw7. (1) FBP Skp2 associates with c-Myc at the promoter and regulates c-Myc target gene transcription. (2) c-Myc is phosphorylated by the Erk family kinases on Ser-62 prior to phosphorylation on Thr-58 by GSK3β. After phosphorylation, c-Myc interacts with Fbw7α for destruction. (*D*) Cyc E is also regulated by Skp2 and Fbw7. (1) The free Cyc E is targeted by Skp2 and Cul3-containing E3 ligases and the Cyc E bound by Cdk2 is targeted by Fbw7α. (2) The ubiquitination of Cyc E is mediated by a two-step activation mechanism. $SCF^{Fbw7\alpha}$ complexes and the isomerase Pin1 are first required for the isomerization of the Cyc E CPD. Cyc E is then translocated into the nucleolus where $SCF^{Fbw7\gamma}$ complexes mediate its ubiquitination. (*E*) The FBP β-TrCP mediates the degradation of Emi1, an inhibitor of the APC/C complex, Cdc25A, which dephosphorylates Cdk1 and promotes its activity, and Wee1A, a kinase that inhibits the function of Cdk1. Wee1A is also targeted by another FBP Tome-1, which is targeted by the APC/C complex during the G1 phase.

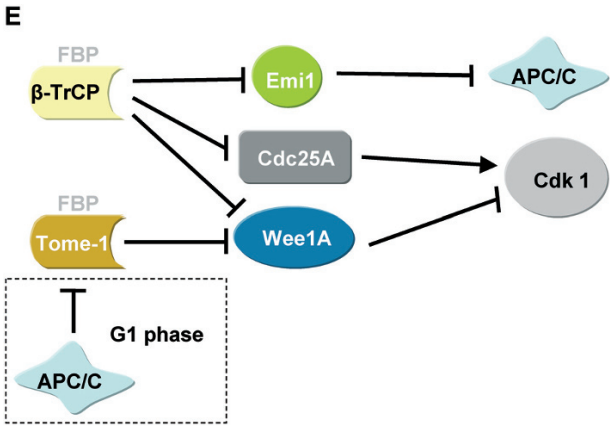

Figure 2. *(Continued)*

phase. It is now commonly believed that Skp2 is the key player in mediating p27 degradation and regulates the G1-S transition by this action.

Recently, Nakayama and colleagues have shown that Skp2 is also involved in the inhibition of Cdk1 (Cdc2), a kinase that functions to promote mitotic entry [62, 63]. Whereas extra centrosomes were seen in *Skp2*$^{-/-}$ single knock-out mice, this replication phenotype was not observed in cells of the *Skp2*$^{-/-}$*; p27*$^{-/-}$ mice, suggesting that the accumulation of p27 during the G2 phase is responsible for the replication defects. It is proposed that p27 associates with Cdk1 and that p27 accumulation results in the reduction of Cdk1 activity in the *Skp2*$^{-/-}$ mice. Therefore, Skp2-mediated p27 degradation is important not only for the G1-S but also for the G2-M transition via tuning the activities of Cdk2 and Cdk1, respectively (Fig. 2B).

Skp2 is considered to be a growth promoter by regulating the tumor suppressor proteins p27, p21, p57, and p130, and cells have laborated multiple strategies to regulate Skp2 activity. For example, transcript levels of Skp2 are cellcycle regulated via E2F factors [64, 65]. Skp2 itself is a target for Cul1-based SCF complexes and its protein levels are controlled through an autocatalytic mechanism during G0 and G1 phases [42]. Moreover, Skp2 is protected from the autoubiquitination by interacting with the Cdc kinase subunit 1 (Cks1), a regulatory factor that binds to Cdks and is essential for the phosphorylation of some cell cycle components [66]. Interestingly, in the context of the SCF^{Skp2} complex, interaction between Cks1 and Skp2 is required for the ubiquitination of p27 *in vitro*, suggesting that Cks1 serves as a cofactor for the SCF^{Skp2} complex [67]. Furthermore, the anaphase-promoting complex or cyclosome (APC/C) complex, and its coactivator Cdh1 directly target Skp2 for degradation, providing a means to regulate the activity of Skp2 during G1 progression (Fig. 2B) [68, 69].

Skp2 and Fbw7 target c-Myc degradation via distinct mechanisms

In addition to p27, Skp2 also regulates the stability of the protein c-Myc, a basichelix-loop-helix/leucine zipper (bHLH/Zip) transcription factor [70, 71]. Heterodimers of c-Myc and the other bHLH/Zip protein Max bind to a consensus E-box sequence essential for gene expressions involved in cellular growth and differentiation. The transactivation domain of c-Myc consists of two conserved regions, Myc box 1 (MB1) and MB2. Although two phosphorylation sites in MB1 were implicated in c-Myc stability [72, 73], Skp2 interacts with the MB2 domain independently to mediate the ubiquitination of c-Myc [71]. It is conceivable that Skp2-mediated degradation of c-Myc is inhibitory for the G1-S transition. Surprisingly, however, Skp2-mediated c-Myc ubiquitination does not prevent cells from entering the S phase. Rather, Skp2 serves as a transcription coactivator which dramatically increases c-Myc transactivation activity and stimulates S phase entry. Moreover, Skp2 and c-Myc are both localized in the nucleus where Skp2 and subunits of the proteasome associate with a c-Myc-targeted promoter [71]. This piece of evidence further demonstrates that Skp2 is involved in inducing the transcriptional activity of c-Myc (Fig. 2C).

The primary degradation mechanism of c-Myc was established when the FBP Fbw7 was discovered to bind c-Myc directly, and this interaction is a phosphorylation-dependent event [72, 73]. Unlike Skp2, Fbw7 promotes the degradation of c-Myc by targeting the MB1 domain. c-Myc is phosphorylated on Ser-62 by Erk family kinases before the kinase GSK3β acts on the residue Thr-58 for the Fbw7 recognition (Fig. 2C) [74]. Thr-58 mutations were seen frequently in a number of human cancer cells where c-Myc protein levels were elevated, suggesting that this residue is critical for c-Myc protein stability [75]. Thus, Fbw7-mediated c-Myc destruction is implicated in tumorgenesis and cell growth.

To summarize, the growth factor c-Myc is regulated by two FBPs, Skp2 and Fbw7. Whereas Skp2 works to stimulate the transactivation activity of c-Myc, Fbw7 mainly determines c-Myc protein levels.

Skp2 and Fbw7 target different pools of Cyclin E

Cyclin E forms a complex with Cdk2 to promote the G1-S transition and ectopic Cyclin E expression has caused premature S phase entry [76, 77]. Interestingly, free Cyclin E is regulated differently from the Cyclin E bound to Cdk2. A type of E3 ligase based on the scaffold protein Cul3 has been shown to be essential for the protein stability of free Cyclin E in a phosphorylation-independent manner [78]. Furthermore, free phosphorylated Cyclin E is directly tar-

geted by Skp2 for ubiquitination both *in vitro* and *in vivo* (Fig. 2D) [62, 79]. Collectively, these results confirm that Skp2 plays key roles during the cell cycle by mediating the destructions of substrates p27, c-Myc, and Cyclin E (Fig. 2A).

In contrast, protein levels of Cyclin E bound to Cdk2 are regulated by the SCF^{Fbw7} complex (Fig. 2D). While all three Fbw7 isoforms are involved in Cyclin E turnover, details of the mechanisms remain elusive. One current model suggests that when both Thr-380 and Ser-384 at the +4 position of the Fbw7-interacting CPD are phosphorylated, Cyclin E is targeted by the $SCF^{Fbw7\alpha}$ complex. However, hypophosphorylated Cyclin E with Ser-384 mutated to alanine is only accessible by the dimeric Fbw7α, suggesting a conditional requirement for Fbw7α dimerization [27]. Dimerization of Fbw7α could potentially recruit additional SCF complexes to ubiquitinate and degrade the substrate, suggesting that differential Fbw7α action provides another level of regulation on substrate recognition and turnover. On a separate note, the Fbw7 substrate c-Myc also consists of a CPD with a negative charge provided by Ser-62 at the +4 position and its ubiquitination is not dependent on the dimeric Fbw7α. Phosphorylation of Ser-62 serves more as a priming event for the subsequent Thr-58 phosphorylation, instead of as a signal for dimeric Fbw7 action as in the Cyclin E scenario.

Sic1p, on the other hand, contains at least nine CPDs to bind Fbw7/Cdc4p [15, 20, 22]. Even though these CPDs have only a low affinity for Cdc4p, the presence of multiple Sic1p CPDs suggests that when gradual phosphorylation of these CPDs has reached a threshold, the recruitment of Cdc4p is induced. Alternatively, the multiple Sic1p CPDs facilitate the recruitment of additional SCF complexes for optimal destruction. Paradoxically, in a recent paper by Hao et al. [20], the crystal structure of the Fbw7-Skp1-Cyclin E complex was solved. Not only were multiple phosphorylation sites on Cyclin E defined, butSic1p was also shown to contain at least three optimal dual-phosphorylated CPDs, a subset of the previously described nine singly phosphorylated weak CPDs [20]. This piece of evidence argues a different mode of Cdc4p action towards the Sic1p substrate.

Fbw7γ/hCdc4γ, distinctively, is involved in a different aspect of Cyclin E degradation. $SCF^{hCdc4\alpha}$ and $SCF^{hCdc4\gamma}$ complexes act sequentially to inactivate Cyclin E *in vitro* [80, 81]. Although both $SCF^{hCdc4\alpha}$ and $SCF^{hCdc4\gamma}$ complexes are capable of mediating the Cyclin E ubiquitination, $SCF^{hCdc4\alpha}$ complexes are especially required for the prolyl isomeration between two proline residues following the crucial threonine (Thr-380) of the Cyclin E CPD. The peptidyl prolyl *cis-trans* isomerase Pin1 binds to Cyclin E and hCdc4α *in vitro* [81], and works in conjunction with $SCF^{hCdc4\alpha}$ complexes to isomerize the bond between the Pro-381 and Pro-382 residues of Cyclin E CPD. Significantly, the prolyl isomeration was found to be prerequisite for the action of $SCF^{hCdc4\gamma}$ complexes. As mentioned above, hCdc4α is localized in the nucleoplasm, while hCdc4γ is found exclusively in the nucleolus [82]. It is tempting to speculate that a relocation event has occurred after the isomeration by Pin1 and $SCF^{hCdc4\alpha}$ complexes in the nucleoplasm. Relocated Cyclin E is ubiquitinated by the $SCF^{hCdc4\gamma}$ complexes in the nucleolus. Thus, isomeration of the CPD might serve as a signal for Cyclin E nucleolar shuffling. To date, CPDs of all characterized mammalian hCdc4 substrates including c-Myc, c-Jun, Notch, and SREBP contain the two prolines (Pro-Pro motif). Nonetheless, the yeast SCF^{Cdc4p} substrates do not contain such motifs. Thus, this two-step inactivation mechanism is probably most applicable for the destruction of SCF^{hCdc4} substrates in mammals (Fig. 2D).

β-TrCP bridges two cell cycle E3 ligases: APC/C and SCF complexes

Two substantial and interrelated types of E3 ligase are known to regulate cell cycle components. A relay constructed by APC/C complexes with cofactors Cdc20 and Cdh1 provides the essential mechanism to modulate mitosis. On the other hand, SCF^{FBP} complexes govern the proteolytic event during G1 and S phases. Interestingly, the activities of both E3 ligases are mutually regulated. First, β-TrCP knockout fibroblasts exhibit cell cycle defects such as extra centrosomes, impaired mitosis, and cell polyploidy [83, 84]. Researchers have shown that these defects are partially due to the accumulation of the specific mitotic factor, Emi1. Emi1 is an inhibitor of APC/C complexes [85, 86]. It is phosphorylated by the mitotic Polo-like 1 kinase (Plk1) and specifically targeted by β-TrCP at the spindle poles during mitosis [87–89]. Once Emi1 is degraded, APC/C complexes carrying Cdc20 are activated and mitosis proceeds accordingly. This example illustrates the action that $SCF^{\beta\text{-}TrCP}$ complexes regulate APC/C complexes indirectly through targeting an APC/C inhibitor (Fig. 2E).

Conversely, the FBP Tome-1 is targeted for destruction by APC/C complexes (Fig. 2E) [90]. Tome-1 controls the stability of the protein Wee1A, a tyrosine kinase that phosphorylates and inhibits Cdk1 required for the onset of mitosis. Thus, Tome-1 triggers mitotic entry by removing the inhibitory effect of Wee1A on Cdk1. Destruction of Tome-1 by APC/C complexes during G1 ensures that Wee1A protein levels persist and mitosis does not start prematurely. In addition, Wee1A is also a β-TrCP target [91]. Though there are no typical β-TrCP recognition phosphodegrons on

Wee1A, two serine residues of Wee1A, Ser-53 and Ser-123, are important for the interaction, and are phosphorylated by two kinases, Cdk1 and Plk1. Wee1A becomes stabilized upon β-TrCP depletion by RNA interference or when the two serine residues are mutated, suggesting that β-TrCP regulates the mitosis onset via Wee1A in addition to Emi1.

Moreover, β-TrCP not only regulates the kinases that affect the activity of Cdk factors, but also exerts control over Cdc25A, a phosphatase that dephosphorylates and activates Cdks. During DNA damage, Cdc25A becomes hyperphosphorylated by checkpoint kinases ChK1 and ChK2. β-TrCP recognizes the hyperphosphophrylated Cdc25A and targets it for degradation to prevent cell cycle progression (Fig. 2E) [92, 93].

FBPs in *Caenorhabditis elegans* synapse development

The development of a nervous system has always been an intriguing issue for researchers to pursue. While the mechanism of nervous system construction continues to be uncovered, the ubiquitin-proteasome pathway has already made a name for itself in the correct wiring of the nervous system [94–97]. Much progress has been made in the understanding of how proteolysis is involved in synapse formation and synaptic plasticity. Here we discuss how FBPs are involved in various steps of synapse development.

FSN-1-containing E3 ligases target ALK and regulate synaptic growth

The RING-domain-containing proteins RPM-1 in *C. elegans*, Hiw in *Drosophila* and Pam in mammals are implicated in the ubiquitin-proteasome pathway [98–100]. RPM-1 localizes in the periactive zone and regulates synapse growth by restricting the size and the number of active zones. The gene *fsn-1* was recovered from a genetic screen for locomotion defect and identified to be a FBP [98]. Mutants of *fsn-1* exhibit similar synaptic defects as *rpm-1*, indicating that FSN-1 and RPM-1 function in the same pathway to regulate synapse growth. Notably, FSN-1 forms an atypical SCF complex with CUL1 and SKR1 (*C. elegans* homolog of Skp1). Nonetheless, RPM-1, instead of the regular RING-domain subunit Rbx1/Roc1, was found to be essential for composing the complex with CUL1, SKR1, and FSN-1. Altogether, these results indicate that synapse development is modulated by the formation of an unconventional E3 ligase in the periactive zones.

A candidate for a potential FSN-1 target is hinted by its intrinsic binding properties. FSN-1 carries the SPRY (spla and ryanodine receptor) domain, a protein-protein-interacting motif proposed to bind to the tyrosine kinase domain. The SPRY domain of FSN-1 was found to interact with a receptor tyrosine kinase homologous to the mammalian anaplastic lymphoma kinase (ALK) [98]. *C. elegans* ALK proteins accumulate ectopically in the periactive zones of the head regions in *fsn-1* and *rpm-1* mutants. Moreover, the *fsn-1* synaptic defects in the larval stage are suppressed by a *C. elegans* ALK mutant. Thus, FSN-1 likely functions as a substrate adaptor in a non-classical SCF complex constituted by SKR1, CUL1, and RPM-1 to degrade ALK in the periactive zones (Fig. 3A) [98].

LIN-23 is essential for cell proliferation, axonal patterning, and GLR-1 receptor abundance

The *C. elegans* FBP LIN-23 was first identified in a screen for mutants in post-embryonic cell divisions [101]. Analyses showed that loss-of-function *lin-23* mutants exhibit overproliferation defects. Later, another screen seeking mutants altering axonal patterning revealed an allele of *lin-23*, *lin-23*^{ot1}, which contains a missense mutation in its C-terminal tail [102]. In *lin-23*^{ot1} mutant worms, axonal anatomy and outgrowth are abnormal, while cell cycle progression remains unaffected. The C-terminal tail of LIN-23 constitutes a PAPP domain which interacts with SH3 domain-containing proteins. Mutations in the C-terminal tail region could potentially disrupt protein-protein interaction between LIN-23 and an unknown factor, resulting in the dysregulation of axonal outgrowth by LIN-23. Thus, LIN-23 is proposed to function in cell cycle control and axonal patterning. Although the targets for ubiquitination by LIN-23 in axonal growth are not yet identified, p21CiP/WAF, which inhibits Rho-kinase activity [103], is a potential candidate [102]. Other possible targets are proteins carrying the SH3 domain in the tyrosine receptor kinase and MAPK pathways, since there is a PAPP domain in the C terminus of LIN-23. Moreover, *lin-23* was isolated again when searching for genes that regulate the abundance of the glutamate receptor GLR-1 [104]. In the screen, GLR-1 protein visualized with green fluorescent protein (GFP) (GLR-1::GFP) was found to accumulate in the mutants of *lin-23*. Interestingly, ubiquitination of GLR-1 and other unidentified proteins has been reported to be important for the regulation of GLR-1 activities [105, 106]. Owing to the substrate-binding ability of LIN-23, GLR-1 was then thought to be a degradative target for LIN-23. However, the amounts of the ubiquitinated GLR-1 (Ub-GLR-1) were no different between the *lin-23* mutants and the wildtype, suggesting that LIN-23 is not required for the for-

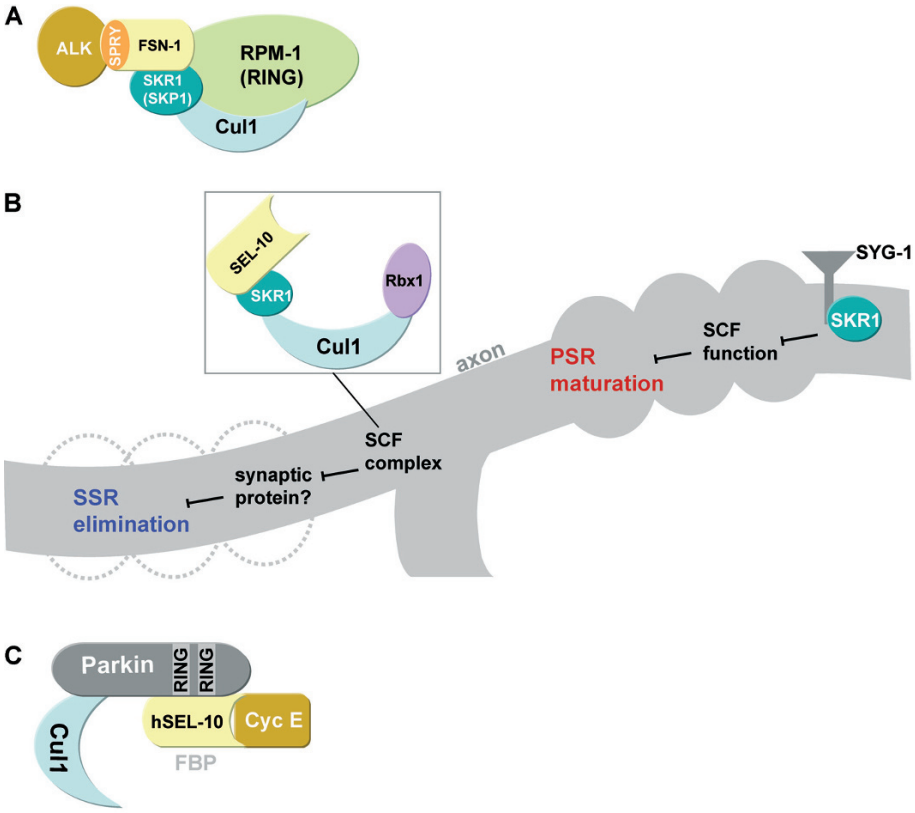

Figure 3. FBPs in synapse development. (*A*) A model of the unconventional SCF^{FSN-1} complex. FSN-1 cooperates with RPM-1 to recruit CUL-1 and SKR-1 and subsequently forms an E3 ligase that targets ALK through the SPRY domain. (*B*) Selective synapse elimination by the SCF^{SEL-10} complex. The SCF^{SEL-10} complex degrades a hypothetical essential synaptic protein in the secondary synaptic region (SSR), and this degradation is disrupted by SYG-1 in the primary synaptic region (PSR). (*C*) A model of the Cul1-Parkin-hSEL-10 complex. Parkin facilitates Cyclin E degradation through the RING domain that recruits hSEL-10.

mation of Ub-GLR-1 conjugates. Instead, implications from the mammalian homolog of LIN-23, β-TrCP, have suggested that the Wnt signaling component BAR-1 (the *C. elegans* homolog of β–catenin) is a direct substrate for LIN-23-containing E3 ligases. In mammals, β-TrCP regulates the Wnt signaling pathway by promoting β–catenin degradation. Similarly, LIN-23 also regulates the protein stability of BAR-1 and the expressions of Wnt target genes in *C. elegans*. Evidently, overexpression of BAR-1 has resulted in increased abundance of GLR-1, mimicking the phenotype observed in the *lin-23* mutants. Therefore, it is proposed that LIN-23 indirectly regulates the abundance of GLR-1 via tuning the stability of BAR-1 and the transcription of Wnt-targeted genes.

SEL-10 is involved in HSNL selective synapse elimination

In *C. elegans,* a hermaphrodite-specific motor neuron, HSNL, required for egg-laying forms specific synapse connection with vulval muscles and VC motor neurons. These synapses form in the primary synapse region (PSR) in the vulval tissue. During development, clusters of synapses are also formed in the secondary synapse region (SSR) anterior to the PSR on the same axon track but they are selectively eliminated and absent in the adult. The specificity of elimination is due to the function of a synaptic adhesion transmembrane protein SYG-1/NEPH1/IrreC that belongs to an immunoglobulin superfamily. SYG-1 is localized only in the PSR, and in *syg-1* mutants, synapses in the SSR are not eliminated while the ones in the PSR are lost [107–109].

A yeast two-hybrid screen has revealed SKR1/Skp1 as one of the SYG-1 interacting partners, and *cul1* and *skr1* RNA silencing experiments suggest that the SCF complex plays a role in SYG-1-regulated synapse elimination. Furthermore, the FBP SEL-10 was found like CUL1 and SKR1 to regulate the SSR synapse elimination and could be the substrate adaptor for this particular SCF complex [107]. Although SEL-10 is localized in both PSR and SSR, the presence of the SYG-1 intracellular domain interferes with the protein-protein interaction between SEL-10 and SKR1. Thus, the regulation is placed at the level of SYG-1 that inhibits the assembly of the SEL-10-containing SCF complex. In summary, the current model proposes that the activity of SEL-10-containing SCF complexes is needed for degrading synaptic components in SSR. Synapses are stabilized in PSR due to the interference activity by SYG-1 present specifically in PSR (Fig. 3B).

hSEL-10 and Parkin regulates neuronal apoptosis

In mammals, hSEL-10 (also known as Fbw7/hCdc4) plays a critical role in the autosomal recessive

Parkinson's disease through its interaction with Parkin, a gene carrying the ubiquitin E3 ligase activity that is mutated in the disease. Parkin contains two RING domains at the C terminus and a ubiquitin homology domain at the N terminus (UHD) [110–112]. The RING domains of Parkin interact with the hSEL-10 F-box domain, and together with CUL1, these components assemble into a distinct SCF complex mediating the degradation of Cyclin E. When cells become deficient in Parkin or the interaction between Parkin and hSEL-10 is disrupted, Cyclin E proteins accumulate and disease-related neuronal apoptosis occurs. Vice versa, overexpression of Parkin protects neurons from apoptosis by controlling Cyclin E protein levels [113]. Notably, the novel Parkin-containing E3 ligase does not include the conventional SCF components such as Skp1 and the E2-recruiting RING protein Roc1/Rbx1, indicating that the FBP hSEL-10 functions differently in distinct E3 complexes (Fig. 3C).

FBPs in *Drosophila* development

Slimb/β-TrCP regulates Hedgehog, Wnt/Wingless, and NF-κB signal transduction pathways

One of the foremost uncovered roles for FBPs focuses on signaling pathways during development. A classical example is the *Drosophila* FBP Supernumerary limbs (Slimb, the homolog of β-TrCP). Jiang and Struhl [114] was first characterized from a screen for mutants altering normal adult patterning and found to negatively regulate the Hedgehog (Hh) and Wnt/Wingless (Wg) signaling pathways. The Hh pathway is mediated through the activity of the transcription factor Cubitus interruptus (Ci) whereas Armadillo (Arm/β-catenin) is the downstream effector for the Wg pathway. In loss-of-function *slimb* mutants, protein amounts of Ci and Arm/β-catenin increase, suggesting that Slimb regulates the stability of these effectors. Adult flies of *slimb* mutants exhibit supernumerary double-anterior wings, and duplicated appendage and limbs, resembling the phenotypes produced by the ectopic expression of Hh or Wg. Due to its intrinsic substrate-binding activity, Slimb is proposed to mediate the degradation of Ci and Arm/β-catenin through the ubiquitin-proteasome pathway, thereby regulating the Hh and Wg signaling activities in tissue patterning [6].

On the other hand, the vertebrate homolog of Slimb, β-TrCP, regulates NF-κB transcriptional activity, which is essential for the inflammatory response and embryonic development [115–117]. β-TrCP targets the inhibitor of NF-κB, IκBα, for ubiquitination and degradation. By doing so, NF-κB is freed from inhibition and enters the nucleus to induce downstream gene expression. IκBα needs to be phosphorylated by the IκB kinase complex prior to the ubiquitination, further implicating an important role of substrate phosphorylation during the FBP-mediated degradation [17].

Archipelago/Fbw7 functions during cell proliferation and tracheal morphogenesis

Most of the developmental roles for Fbw7 have been elucidated in *Drosophila*. The *Drosophila* Fbw7, Archipelago (Ago), was discovered in a screen for genes that affect cell proliferation during eye development [24]. While single-cell size is similar to the wild-type, mutants of *ago* display a growth advantage over adjacent wild-type cells, suggesting that additional rounds of cell divisions occur. Later, the *Drosophila* ortholog of c-Myc, dMyc, was identified *in vivo* as an Ago substrate [118]. Upregulation of dMyc protein levels causes an increase in the fly body and wing sizes, an observation phenocopying the *ago* mutant. Conversely, loss-of-function dMyc mutants result in an opposite phenotype. Moreover, Ago was recently found to regulate tracheal morphogenesis by targeting the Trachealess (Trh) protein and the breathless FGF receptor in the development of the *Drosophila* embryonic tracheal system [119]. To summarize, these results suggest that Ago controls the stability of different substrates to regulate cell proliferation and *Drosophila* tissue development [6].

FBPs in phytohormone responses

Plant hormones (phytohormones) play essential roles in almost every aspect of plant development and physiology. FBPs are involved in plant responses to at least four major phytohormones: auxin, jasmonate (JA), gibberellin (GA), and ethylene. Here we briefly review these different types of phytohormones and discuss how FBPs function during the responses (Fig. 4).

TIR1 is an auxin receptor targeting AUX/IAA degradation

The small indolic compound indole-3-acetic acid (IAA or auxin) regulates plant developmental and physiological processes. Central to the auxin response are changes in gene expression mediated through a family of nuclear proteins called AUX/IAA, which negatively regulate auxin responses. AUX/IAA forms heterodimers with the auxin-specific DNA-binding transcriptional factor AUXIN RESPONSE FACTORs (ARFs) to block their transcription activities. The FBP TRANSPORT INHIBITOR RESPONSE 1

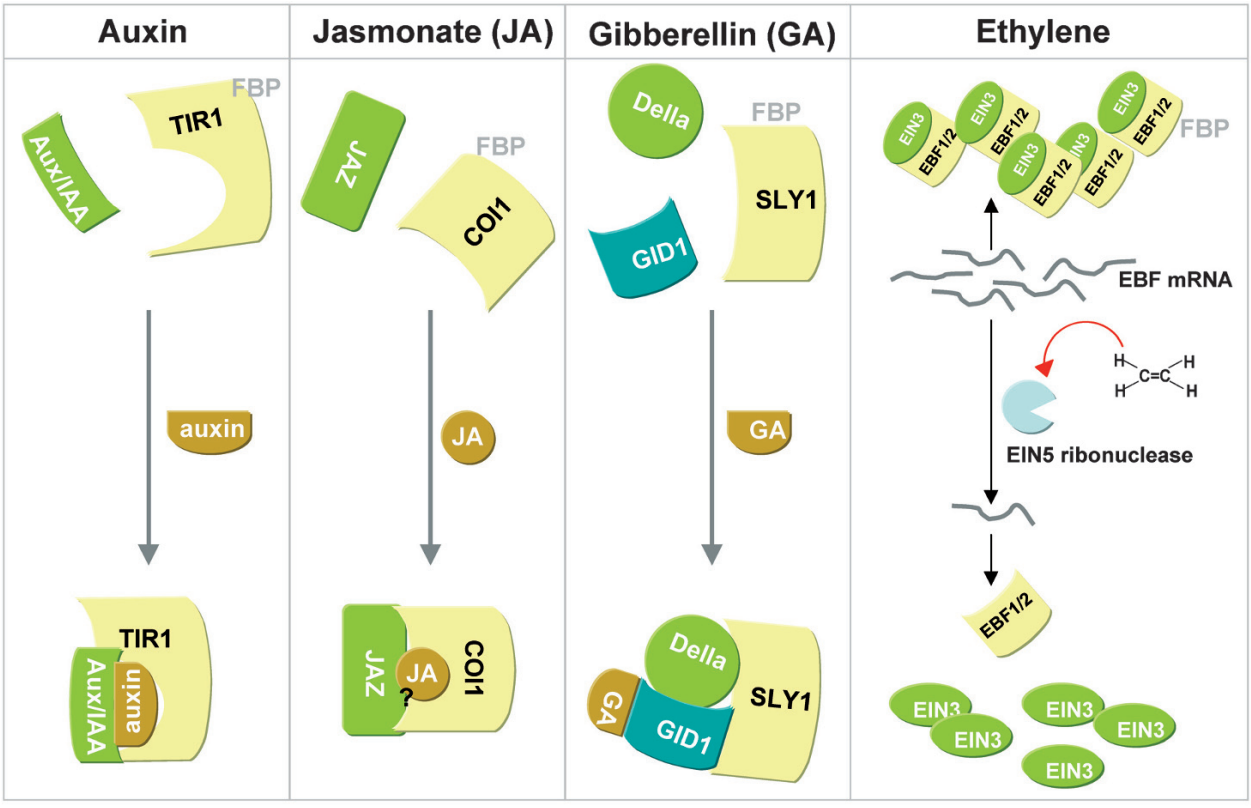

Figure 4. FBPs in plant hormone response pathways. In the auxin pathway, the FBP TIR1 is the auxin receptor. Auxin stabilizes the interaction between TIR1 and the substrate AUX/IAA proteins by filling the gap between them. The JA hormone induces the physical interaction between the FBP COI1 and the substrate JAZ, but the detailed molecular mechanisms remain unclear. In the GA pathway, interaction between the FBP SLY1 and its substrate Della proteins is through the GA-bound GID1 receptor. The gaseous hormone ethylene reduces mRNA levels of FBPs EBF1 and EBF2 through EIN5 ribonuclease. Thus, substrate protein EIN3 accumulates in the presence of ethylene.

(TIR1) regulates auxin-dependent gene expression by degrading AUX/IAA. TIR1 contains 16 degenerate LRRs [120] and physically associates with *Arabidopsis* SKP1 (ASK1), ASK2, and AtCUL1 to form a SCF^{TIR1} complex *in planta* [121]. Thus, when auxin levels increase, auxin-inducible gene expression is derepressed by the TIR1-mediated AUX/IAA proteolysis [122].

Mutations in the AUX/IAA conserved domain II disrupt the TIR1-AUX/IAA interaction and stabilize AUX/IAA proteins, suggesting that an intact domain II is required for AUX/IAA proteolysis [121, 123]. Unlike classical FBPs which recognize substrates through signaling-induced substrate phosphorylation, mutations of potential phosphoylation sites in the AUX/IAA domain II do not increase protein stability [123]. Experiments in a cell-free system also showed that direct addition of auxin into the plant lysates induced rapid TIR1-AUX/IAA interaction and this interaction was not affected by the presence of a kinase inhibitor. Therefore, auxin induces TIR1-AUX/IAA interaction in a phosphorylation-independent manner [124, 125].

Interestingly, TIR1 senses the auxin stimulation by directly binding with the hormone [126, 127]. Structural analysis of TIR1 shows that the LRR domain comprises a surface hydrophobic pocket that binds to both auxin and AUX/IAA proteins [128]. Auxin binding does not induce significant conformational changes of TIR1. Instead, it acts as an adaptor between TIR1 and AUX/IAA to enhance their interaction by filling the cavity at the interface of these two proteins.

TIR1 belongs to a small sub-family of FBPs consisting of seven members, three of which (AFB1, 2, and 3) have been shown to mediate the auxin response as well. Quadruple mutants of *tir1 afb1 afb2 afb3* are completely insensitive to auxin treatment, and exhibit severe embryonic phenotypes similar to the gain-of-function *aux/iaa* mutants [129]. Thus, these four FBPs collectively modulate the plant auxin responses. Another member of this protein family, AFB5, has been shown to mediate the response to a particular synthetic auxin compound, suggesting that different members of this FBP family confer the chemical specificity seen in auxin signaling [130].

COI1 and the substrate JAZ interaction is stimulated by JA

An important player during the JA responses that regulates both stress responses and plant development is the FBP CORONATINE INSENSITIVE 1 (COI1). COI1 associates with AtCUL1, AtRbx1, ASK1 and ASK2 *in planta* [131] and is required for JA-mediated pollen development and activation of defense mechanisms against pests and pathogens [132]. It was recently discovered that COI1 activates transcription of JA-responsive genes by degrading a family of JA ZIM-domain (JAZ) proteins [133, 134]. JAZ proteins are degraded in response to JA treatment, and this degradation is blocked in *coi1* mutants. Experiments in the cell-free system show that JA addition into the plant extract stimulates the physical interaction between JAZ proteins and COI1. Since COI1 belongs to the TIR1 sub-family of FBPs, it will be interesting to define how the interaction between COI1 and JAZ is regulated by JA [134].

SLY1 binds substrate through complex formation with GA receptors

GA promotes important processes of plant growth and development, such as seed germination, elongation growth, and induction of flowering [135]. Two *Arabidopsis* FBPs SLEEPY1 (SLY1) and its paralog SNEEZY (SNE), together with the rice ortholog GIBBERELLIN INSENSITIVE DWARF2 (GID2), are involved in GA signaling [136–139]. Both SLY1 and GID2 interact with ASK1, suggesting that they function within the SCF complex [138, 140]. These FBPs regulate GA responses by degrading a small family of nuclear transcriptional factor DELLA proteins characterized by the conserved N-terminal DELLA domain. In the *sly1* or *gid2* mutants, DELLA proteins accumulate, thereby resulting in dwarf plants insensitive to GA.

Although the DELLA domain is essential for GA-induced degradation [141], it is not required for interaction with the FBPs [137]. In fact, the DELLA domain mediates GA-dependent interactions between DELLA proteins and the GA receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) [142–144], indicating that the interaction with GID1 is required for DELLA protein degradation. Furthermore, a yeast three-hybrid assay shows that the presence of the receptor GID1 strongly enhances the interaction between the FBP SLY1 and the DELLA protein in a GA-dependent manner [142]. Thus, during GA signaling, target recognition by the FBPs is mediated through ligand-receptor complex formation to promote substrate ubiquitination.

DELLA protein SLR1 is phosphorylated in response to GA treatment [138], and the FBP SLY1 also appears to associate with the phospho-DELLA protein better *in vivo* [140]. These results indicate that in contrast to auxin signaling, plants mediate the GA response in a phosphorylation-dependent manner. Irrespective of the unknown details of how GA stimulates DELLA protein phosphorylation, and how important this event is for the interaction among all components, phosphate modification of the substrate is important for the substrate recognition by FBPs in GA signaling.

EBF-promoted EIN3 degradation is prevented by ethylene

The gaseous hormone ethylene regulates plant development and stress response. Ethylene-induced transcriptional responses are positively regulated by the DNA-binding transcriptional factor ETHYLENE-INSENSITIVE 3 (EIN3), and possibly five other EIN-3-like (EIL) factors. In the absence of ethylene, the EIN3 protein is constitutively degraded by two closely related FBPs EIN3 BINDING F-BOX Protein 1 (EBF1) and EBF2 [145–147]. Ethylene treatment induces the nuclear accumulation of EIN3, a phenotype also seen in *ebf1 ebf2* double mutants. Thus, during the ethylene response, FBP-mediated degradation of EIN3 is prevented by hormone treatment. This mode of regulation differs from the other three phytohormone pathways, in which hormones promote, rather than inhibit, the FBP-mediated protein degradation.

Although both EBF1 and EBF2 promote EIN3 degradation in the absence of ethylene, detailed analyses of the single-mutant *ebf1* and *ebf2* reveal that EBF1 and EBF2 play temporally distinct roles. Whereas EBF1 is important during the initial phase of signaling, EBF2 functions more prominently during later stages of the response and the resumption of growth after ethylene removal [148]. Interestingly, the transcript levels of EBF1 and EBF2 are negatively regulated by the exoribonuclease EIN5, the plant ortholog of XRN1, a 5′ to 3′ exoribonuclease involved in mRNA decay in *S. cerevisiae* [149, 150]. *EBF1* and *EBF2* mRNA levels are elevated in plant *ein5* mutants, which are ethylene insensitive. The turnover rate of *EBF1* and *EBF2* mRNA is however unaltered, suggesting that EIN5 might modulate *EBF1* and *EBF2* indirectly by affecting their *EBF1* and *EBF2* transcription [150]. To date, it is still unclear how *EIN5* activity is regulated in response to ethylene treatment.

FBPs in plant organ formation

UFO and MAX2 are essential for flowering development and shoot branching

Several FBPs are involved in development of plant organs such as flowers, shoots, and roots. The FBP UNUSUAL FLOWER ORGANS (UFO) in *Arabidopsis* and its ortholog FIMBRIATA (FIM) in *Antirrhinum* are involved in flower development by controlling the expression of organ identity genes [151, 152]. Protein-protein interaction with ASK1 and ASK2 and genetic interaction with AtCUL1 suggest that UFO functions in the SCF context to regulate flower development, although the substrate has not been identified [152, 153]. The other FBP MAX2 is involved in axillary shoot development. In *max2* mutant plants, the shoot is highly branched [154, 155]. MAX2 interacts with ASK1 and AtCUL1 *in planta*, indicating that MAX2 inhibits shoot branching by mediating protein degradation of unidentified positive regulators in shoot branching.

ARABIDILLO, CEG and VFB are required for lateral root formation

As described above, the *Drosophila* Arm and its mammalian homolog β-catenin play important roles during animal development. Interestingly, two F-box proteins, ARABIDILLO-1 and 2, that include Arm repeats, regulate lateral root branching in *Arabidopsis* [156]. Fewer lateral roots are formed in *arabidillo-1 arabidillo-2* double-mutants, and overexpression of ARABIDILLO-1 results in increased lateral roots. Despite the fact that auxin signaling is also required for lateral root formation [120], both wild-type and *arabidillo-1 arabidillo-2* double mutant plants show similarly increased lateral root density in response to the auxin treatment, suggesting that ARABIDILLO-1 and 2 are not directly involved in auxin signaling during lateral root development.

While ARABIDILLO-1 and 2 positively regulate lateral root formation, another FBP, CEGENDUO (CEG), functions distinctively in this developmental process [157]. In contrast to ARABIDILLO-1 and 2 which function independently from the auxin pathway, lateral root formation in *ceg* mutant plants is hyposensitive to auxin. Furthermore, auxin treatment enhances *CEG* transcript levels, suggesting that CEG might be involved in a feedback regulation in response to auxin during lateral root development.

Recently, a new family of FBPs, VIER F-BOX PROTEINE (VFB) 1 to 4, has also been shown to regulate lateral root formation [141]. *vfb4* RNAi mutant plants exhibit fewer lateral roots, and in the case of quadruple mutants, a general delay in plant growth is also observed. Microarray and *in vivo* analyses show that the expression of a number of auxin-responsive genes is reduced in *vfb* mutants. However, the auxin-dependent growth responses and the turnover rate of the AUX/IAA proteins remain unaffected, suggesting that VFB mediates the expression of auxin-responsive genes in a way different from the FBP TIR1.

SLF proteins regulate pollen self-incompatibility by degrading the non-self S-RNase

Self-incompatibility (SI) in many flowering plants prevents inbreeding, thus generating genetic diversity within a species. The SI response allows plant pistils to distinguish self-pollen from non-self-pollen, and thus to inhibit fertilization from self-pollen [158]. In the Solanaceae, Scrophulariaceae, and Rosaceae, SI is mediated by two types of highly polymorphic genes at the S-locus: the *S-RNase* gene encodes the pistil determinant, and the clusters of *S-Locus F-box* (*SLF or SFB*) genes encode the pollen determinants [159, 160]. During fertilization, the S-RNase secreted by the pistil is incorporated into the pollen tube to act as a cytotoxin to hydrolyze the pollen RNA. Thus, in order to promote fertilization with non-self pollen in the pistil, the non-self S-RNase has to be degraded. The FBP AhSLF-S2 interacts with S-RNase proteins as well as SKP1- and CUL1-like proteins in *Antirrhinum*, a member of the Scrophulariaceae [159, 161]. Studies of the FBP protein Pi SLF in *Petunia inflata* (Solanaceae) further show that SLF proteins interact better with the non-self S-RNase than with self S-RNase [162], suggesting that SLF might preferentially target non-self S-RNase for degradation. Moreover, S-RNase is ubiquitinated by the compatible pollen extract [163], supporting the model that pollen SLF promotes fertilization by degrading the non-self S-RNase.

According to the current F-box-mediated S-RNase degradation model, the absence of FBPs should lead to universally incompatible pollen. However, it has been found that SLF proteins carrying deletion or truncation at the C-terminus associate with self-compatibility, a phenotype opposite to that predicted by the current model [164, 165]. Thus, more detailed analyses are needed to further understand the roles of FBPs in self-incompatibility.

FBPs in light signaling and circadian rhythm

Organisms maintain their own oscillators by sensing external environmental cues such as seasonal changes and light signaling. Here we discuss mechanisms in which FBPs are involved in light sensing and how FBPs modulate the circadian clocks.

EID1 and AFR are involved in phyA-dependent light signaling

Phytochromes are the photoreceptors that allow plants to sense the red/far-red light as an environmental cue. This photoreceptor group comprises five members, phytochromes A–E (phyA–E) in *Arabidopsis*. Two FBPs, EMPFINDLICHER IM DUNKELROTEN LICHT1 (EID1) and ATTENUATED FAR-RED RESPONSE (AFR), are involved in phyA-dependent light signaling. EID1 is a nuclear FBP with a leucine zipper domain [166]. It interacts with ASK and AtCUL1 in plant cells, suggesting that EID1 is a component of the SCF complex [166, 167]. Light sensitivity is highly enhanced in *eid1* mutants, suggesting that EID1 most probably targets the degradation of an activated component of the phyA signaling pathway, although its identity is still unknown [166].

The FBP AFR was identified in an RNAi screen for E3 components in light signaling or the circadian clock [168]. AFR is composed of an N-terminal F-box domain required for interaction with ASK1, and two C-terminal Kelch repeats which presumably mediate protein-protein interaction through their β propeller structures. The mRNA levels of AFR are controlled by both the circadian clock and far-red light. In *afr* RNAi transgenic plants, light responsiveness is attenuated, suggesting that AFR promotes the degradation of a yet-to-be-identified repressor in phyA signaling.

ZTL sets the plant circadian clock

ZEITLUPE (ZTL) is the first identified FBP in 2 plant circadian system. In *ztl* mutants, the free-running period of clock-controlled gene transcription is increased [169]. ZTL associates with ASK1, AtCUL1 and AtRBX1 to assemble into an SCF complex [170] that regulates the circadian clock by degrading TIMING OF CAB EXPRESSION 1 (TOC1), a central clock protein [171]. TOC1 is a member of the PSEUDO-RESPONSE REGULATOR (PRR) family proteins. Recently, another member of the PRR family, PRR5, has also been shown to be targeted for degradation by ZTL [172].

ZTL includes an N-terminal LIGHT, OXYGEN OR VOLTAGE (LOV) domain, which has been shown to bind to the chromophore flavin mononucleotide and to function as a blue light sensor [173]. Although *ztl* messenger RNA is constitutively expressed, ZTL protein levels oscillate. The stabilization of ZTL protein is through an interaction with GIGANTEA (GI) [174], a putative transmembrane protein involved in circadian rhythms [175, 176]. This interaction between GI and ZTL is enhanced by light-sensing activity of the LOV domain [174]. Thus, the FBP ZTL functions as a blue-light photoreceptor, and becomes stabilized by light-enhanced interaction with GI for TOC1 degradation (Fig. 5A).

FKF1 regulates plant flowering

Flowering in many species is controlled by a circadian clock and by changes in day length (or photoperiod), an environmental cue associated with seasonal progression [177]. In the *Arabidopsis* photoperiodic flowering pathway, increased day length enhances the daytime expression of the transcriptional factor CONSTANS (CO) to promote flowering. The FBP flavin-binding, KELCH REPEAT F-BOX1 (FKF1) plays an essential role in regulating CO daytime expression [173] by degrading the transcriptional repressor CYCLING DOF FACTOR 1 (CDF1) [173, 178]. CO daytime expression reaches its maximum when CDF1 is degraded by FKF1 in a light-dependent manner. FKF1 belongs to a family of three proteins including ZTL and LOV KELCH PROTEIN2 (LKP2) [179]. All three proteins contain an N-terminal LOV domain, a central F-box domain, and C-terminal Kelch repeats.

Recent studies also show that FKF1 is sensitive to blue light and interacts with GI through its LOV domain. The FKF1-GI interaction is important for the timing of CO daytime expression [180]. In *fkf1* or *gi* mutant plants, CDF1 is stabilized and the flowering timing is delayed [178, 180]. Biochemical analyses further showed that FKF1, GI and CDF1 proteins associate within the *CO* chromatin structure, suggesting that FKF1-GI complexes directly target DNA-bound CDF1 to relieve transcriptional repression of *CO* (Fig. 5A) [180].

Slimb mediates PER degradation during constant darkness

Animal circadian rhythms are regulated by a central clock located in the suprachiasmatic nucleus (SCN), where transcription factors CLOCK and BMAL1 form heterodimeric complexes to induce the expression of circadian genes. The activity of CLOCK-BMAL1 complexes is negatively regulated by the expressions of their own targets, Period proteins PER1 and PER2 and cryptochromes CRY1 and CRY2. PER and CRY proteins form heterodimers and translocate into the nucleus to inhibit CLOCK-BMAL1 complexes (Fig. 5B). In *Drosophila*, similar regulatory loops are utilized with minor tunings. CLK, a homolog of CLOCK, binds to CYCLE (CYC) and forms CLK-CYC heterodimers equivalent to the mammalian CLOCK-BMAL1 complexes. The *Drosophila* Period (PER) protein binds to Timeless (TIM) instead of CRY and translocates into the nucleus as PER-TIM heterodimers to inhibit

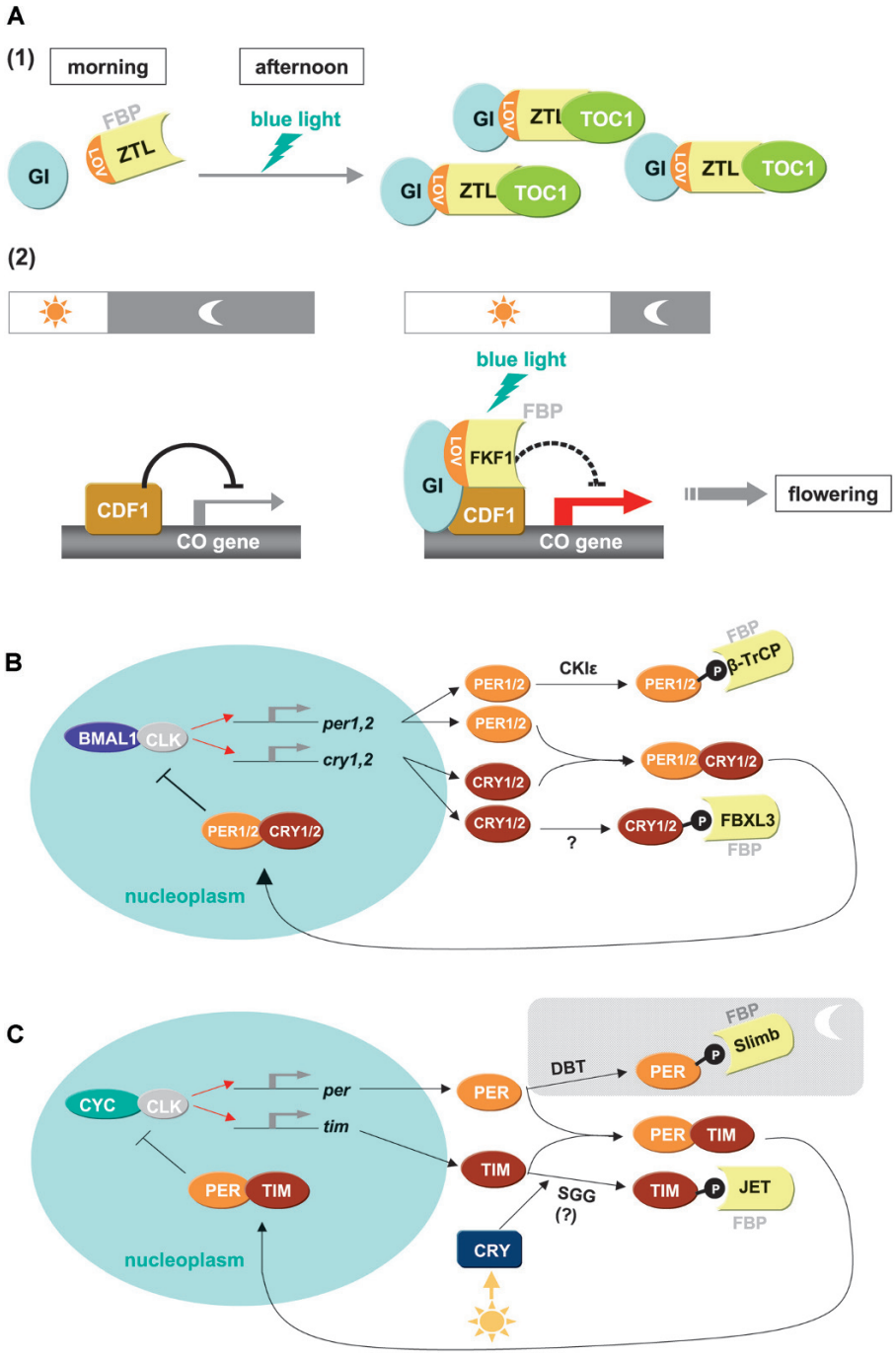

Figure 5. FBP in light signaling and circadian rhythm pathways. (*A*) The FBPs ZTL and FKF1 function as blue-light sensors for GI interaction. (1) ZTL interacts with GI through the blue light-sensing LOV domain. ZTL-GI association stabilizes both proteins, allowing more ZTL to target TOC1 for degradation in the plant circadian rhythm. (2) FKF1 associates with GI through the LOV domain. The FKF1-GI complex binds to and degrades substrate CDF1 on the CO chromatin to promote CO expression and flowering. (*B*) The circadian mechanism in mammals. The BMAL1/CLK heterodimers are responsible for the transcription of *per1/2* and *cry1/2* genes. PER and CRY proteins form heterodimers and translocate into the nucleus to inhibit BMAL1-CLK-dependent transcription. PER1/2 proteins are phosphorylated by the kinase CKIε and targeted by the FBP β-TrCP, whereas the CRY1/2 proteins are ubiquitinated by FBXL3. The kinases for CRY1/2 phosphorylation are not clear. (*C*) The circadian mechanism in *Drosophila*. The CYC-CLK heterodimers in the nucleus activate the transcription of *period* (*per*) and *timeless* (*tim*). PER and TIM then translocate as heterodimers and inhibit CYC/CLK-mediated transcription. PER is phosphorylated by Doubletime (DBT) prior to the recognition by the FBP Slimb during constant darkness. TIM undergoes phosphorylation by SHAGGY (SGG). It is also targeted by the FBP JETLAG (JET) in a light-dependent manner.

CLK-CYC transcription activity (Fig. 5C) [181–183].

Several reports have described a role for the FBP Slimb in circadian rhythm by regulating the stability of PER. Genetically, *slimb* mutants were shown to be behaviorally arrhythmic. This disruption in rhythmicity can be rescued by the expression of Slimb specifically in the clock neurons. PER proteins in the *slimb* mutants were shown to be hyper-phosphorylated in constant darkness. It was shown that phosphorylation of PER is mediated by Doubletime (Dbt), the *Drosophila* homolog of casein kinase Iε (CKIε) [184–186]. Further analysis shows that Slimb binds to the hyper-phosphorylated PER preferentially and stimulates its degradation. A similar mechanism was shown in a third study, in which the mammalian PER1 protein is phosphorylated by CKIε and degraded in a β-TrCP-dependent manner (Fig. 5B, C) [187].

JET-regulated TIM degradation is light dependent

In *Drosophila*, the photo-sensing factor CRY interacts with TIM, thereby transmitting information to the central clock in a light-dependent manner [188, 189] Nonetheless, how exactly the messages are conveyed remains unclear. It was found that phosphorylation of TIM is required for its degradation and ubiquitination in cultured cells [190], and the phosphorylation of TIM was found to be mediated by the fly homolog of glycogen synthase kinase 3 (GSK3), SHAGGY (SGG) [191]. It will be interesting to determine whether TIM phosphorylation by SGG is required for its ubiquitination and degradation. Recently, researchers have found that the FBP JETLAG (JET) mediates the ubiquitination of TIM upon photo-activation. When the flies perceive light, CRY binds to TIM, leading to the subsequent ubiquitination and degradation of TIM [192, 193]. Mutant *jet* flies are rhythmically insensitive in constant light and the abnormal rhythmic behaviors can be rescued by expression of JET in clock-specific neurons. Furthermore, *jet* mutant flies exhibit reduced responses to light pulses and retardation in light-entrained TIM degradation. Thus, depending on the external cues, *Drosophila* applies different programs to regulate the rhythmic outputs. In the dark, clock-controlled mechanisms utilize the FBP Slimb for mediating the degradation of PER. Alternatively, in the light entrainment pathway, photo-activation of CRY triggers JET to degrade TIM (Fig. 5C).

FBXL3 promotes CRY degradation and regulates circadian hours

In the mammalian clock system, the negative feedback loop mediated by PER-CRY heterodimers is prohibited via CRY degradation. Three separate studies have implicated the FBP FBXL3 in this process [194–196]. By forward genetic ENU mutagenesis screens, animals carrying either alleles of FBXL3, *Overtime* (*Ovtm*) or *After hours* (*Afh*), were found to exhibit longer period behavioral rhythms. Both alleles were mapped to the LRR regions of FBXL3 which disrupts the interaction with CRY proteins, suggesting that FBXL3 mediates the ubiquitination and degradation of CRY proteins. In loss-of-function *FBXL3* mutants, CRY proteins are stabilized and the repression of CLOCK-BMAL1 activity is enhanced. These results indicate an essential function of FBXL3 in directing CRY degradation in circadian rhythm (Fig. 5B).

FBPs in viral and bacterial infection

β-TrCP interacts with the HIV-1 viral protein U (Vpu) and triggers CD4 degradation

Viruses promote their own replication via various strategies to subvert the cellular functions in host cells. Ubiquitin-mediated degradation of domestic targets is one of the means viruses have taken to disrupt host defenses, allowing further cell invasion. For example, the human immunodeficiency virus type-1 (HIV-1) viral protein U (Vpu) contributes to virulence in two ways. First, Vpu enhances the release of retroviral particles from the infected human cells to boost infection efficiency. Vpu also triggers degradation of the surface receptor CD4 in the endoplasmic reticulum (ER) via a proteasomal-dependent pathway. It is known that β-TrCP interacts with Vpu and forms a ternary complex with CD4. Vpu contains a DS_{52} $GXXS_{56}$ motif which is constitutively phosphorylated and recognized by β-TrCP. The two conserved serine residues are both required for the degradation of CD4, but Vpu is unlikely to be the substrate for β-TrCP. By interaction with Vpu, β-TrCP is recruited to the membranes for CD4 degradation and for sequestration so that it is prevented from degrading its natural substrates like β-catenin and IκBα. To summarize, β-TrCP is involved in HIV-1 viral infection by participating in the degradation of the viral receptor CD4 via interaction with the adaptor Vpu [197–199].

Fbw7 interacts with the SV40 large T antigen

Cells infected with SV40 are highly transformed and activities of host tumor suppressors like p53 and pRb are strikingly affected. Infection mechanisms of Simian virus 40 (SV40) are similar to the HIV-1 Vpu in that SV40 large T antigen (T Ag) also interacts with Fbw7. T Ag contains a sequence mimicking the consensus Fbw7 CPD at the C terminus for the recognition. Nonetheless, instead of degrading T Ag, Fbw7 hinders the degradation of its natural substrate such as Cyclin E by competitive binding [200].

P0 and VirF of plant pathogens attack host defense mechanisms

Post-transcriptional gene silencing is one of the antiviral defense mechanisms employed by plants [201]. To counter this defense response, many plant viruses encode silencing suppressor proteins. One of these is the polerovirus P0, an FBP. P0 interacts with the *Arabidopsis* ASK1 through its F-box motif [202]. Point mutations in the F-box motif that abolish the P0-ASK interaction lead to reduced suppressor activity and diminished virus pathogenicity. Furthermore, knock-down of the SKP1 homolog in *Nicotiana benthamiana* renders plant resistant to the polerovirus

infection, supporting the model that P0 is part of the SCF complex and targets the host post-transcriptional gene silencing machinery. Recently, two groups have found that P0 promotes the degradation of ARGONAUTE1 (AGO1), a key component of the RNA-induced silencing complex (RISC) [203, 204]. P0 physically interacts with AGO1 in the nucleus in *Arabidopsis*. Ectopic expression of P0 induces AGO1 protein decay *in planta* [203, 204] and transcriptional upregulation of several miRNA-target genes [204]. Thus, P0 suppresses host gene silencing mechanisms by targeting AGO1 for degradation.

In addition to virus, the pathogenic bacterium *Agrobacterium tumefaciens* also attacks host cells through SCF-mediated protein degradation. *Agrobacterium* secrets the virulent protein VirF, which is then exported into plant cells to aid the infection process. VirF, an FBP, binds to the host plant homolog of SKP1 [205] and destabilizes the host target protein VIP1 in an SKP1-dependent manner, indicating that VirF mediates VIP1 degradation in the SCF context [206]. During the infection process, it is suggested that VIP1 degradation in the host cells is required to facilitate the infection process [206].

Fbs1 in glycoprotein quality controls

In the E. R., folding of proteins determines their future routes. Correctly folded proteins assisted by ER chaperones are transported out to the Golgi complex for subsequent action, whereas misfolded ones are kept in the E. R., retrotranslocated into the cytosol, and finally degraded by the ER-associated degradation pathway ERAD. In this regard, the ER-resident high-mannose oligosaccharides, essential for protein folding, trafficking and sorting of the glycoproteins, have emerged to be a central means of regulating the abundance of misfolded proteins, thereby ensuring that glycoprotein quality control is achieved.

Recently, researchers have grouped together a subset of five FBPs by their homologous sugar-binding domain at the C terminus. Among them, Fbs1 is the most-characterized due to its role in mediating the degradation of N-linked glycosylated proteins (N-glycans). Fbs1 was first isolated in a screen for proteins bound to various glycoproteins in mouse brain extracts [207]. Being a component of SCF E3 ligases, Fbs1 degrades glycosylated substrates such as pre-integrin β1 and the extracellular N-terminal domain of NMDA subunit NR1 in the cytosol [207, 208]. On the other hand, Fbs1 alone is involved in preventing the aggregation of glycoproteins *in vitro* and acts to clear aberrant glycoproteins in neuronal cells [209]. Moreover, Fbs1-deficient mice are defective in inner ear homeostasis and exhibit cochlear degeneration [210]. Insights into how Fbs1 and the related proteins recognize sugar chains are provided by pull-down analysis using various oligosaccharides *in vitro*. For efficient Fbs1 binding, a sugar peptide like $Man_{3-9}GlcNAc_2$ needs to be present in the substrate. Crystallographic studies have indicated that the sugar-binding domain of Fbs1 interacts with the innermost chitobiose (GlcNAc-GlcNAc) in N-glycans [211, 212]. Since the innermost chitobiose is normally not exposed in the structure, Fbs1 is hypothesized to interact better with N-glycans in a denatured condition. Upon protein denaturation, the innermost chitobiose becomes exposed and is used as a signal for Fbs1 to recognize the unfolded proteins in the cytosol after retrotranslocation from the ER. Therefore, high-mannose oligosaccharides serve not only as tags for the recognition by the ER quality control system, but also as degradation signals in the cytosol demonstrated by Fbs1.

Perspective

Immense progress has been made during a single decade in understanding the functions of large FBP families in animals and plants. Originally proteins carrying an uncharacterized F-box motif, FBPs have demonstrated their own importance by functioning in a variety of cellular settings. Studies have shown that either working in concert with the SCF complex or alone, FBPs possess both proteolytic and non-proteolytic functions. With the large number of uncharacterized FBPs that await analysis in model organisms, it is reasonable to speculate that FBPs function in yet-to-be-characterized molecular and cellular pathways we do not yet understanding. With the tremendous effort now being made by the research community, more such discoveries are sure to be made in the near future. Insights from these findings will surely benefit advances in agricultural biotechnology and medical therapy for human diseases.

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