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F-box proteins: the key to protein degradation

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Summary

The eukaryotic protein degradation pathway involves the ubiquitin (Ub) modification of substrates targeted for degradation by the 26S proteasome. The addition of Ub, a process called ubiquitination, is mediated by enzymes including the E3 Ub ligases which transfer the Ub to targeted substrates. A major type of E3 Ub ligases, the SCF (Skp–Cullin–F-box) complex, is composed of four major components: Skp1, Cul1/Cdc53, Roc1/Rbx1/Hrt1, and an F-box protein. The F-box component of the SCF machineries is responsible for recognizing different substrates for ubiquitination. Interaction with components of the SCF complex is mediated through the F-box motif of the F-box protein while it associates with phosphorylated substrates through its second protein–protein interaction motif such as Trp–Asp (WD) repeats or leucine-rich repeats (LRRs). By targeting diverse substrates, F-box proteins exert controls over stability of proteins and regulate the mechanisms for a wide-range of cellular processes. Here we discuss the importance of F-box proteins by providing a general overview and examples of how F-box proteins function in various cellular settings such as tissue development, cell proliferation, and cell death, in the modeling organism *Drosophila*.

Fundamental cellular activities in eukaryotes are organized in a coordinated fashion through complex interrelating networks maintained largely by generation and destruction of proteins. An important index for accomplishing this cellular balance is the dynamic protein levels. By regulated processes like synthesis and degradation, protein turnover are under delicate temporal and spatial controls. In particular, the regulation of protein degradation is of great importance and although it has been studied less than protein synthesis, some important principles have emerged. Proteins targeted for degradation by the proteasomal pathway are covalently attached to a small molecule ubiquitin (Ub), a process called ubiquitination [1–3]. Ubiquitination plays the regulatory role in the Ub-proteasomal degradation pathway involving three major enzymes: E1 activing enzyme, E2 conjugating enzyme, and E3 ubiquitin ligases [Figure 1a, 4, 5]. E1 and E2 enzymes are responsible for activating and conjugating the Ub moieties, respectively, whereas E3 ligases are important for transferring Ub moieties from the E2 enzyme onto the recognized substrates destined for destruction by the 26S proteasome [4–7]. How do E3 ligases define their substrates? How are the substrate proteins chosen to be discarded? These questions remain intriguing issues within the field of protein degradation.

Two types of E3 ligases, HECT-domain and RING-domain type, utilize different mechanisms to transfer the Ub adducts to substrates [4, 7–9]. Whereas RING-domain E3s transfer Ub directly onto substrates from the E2 enzymes, HECT-

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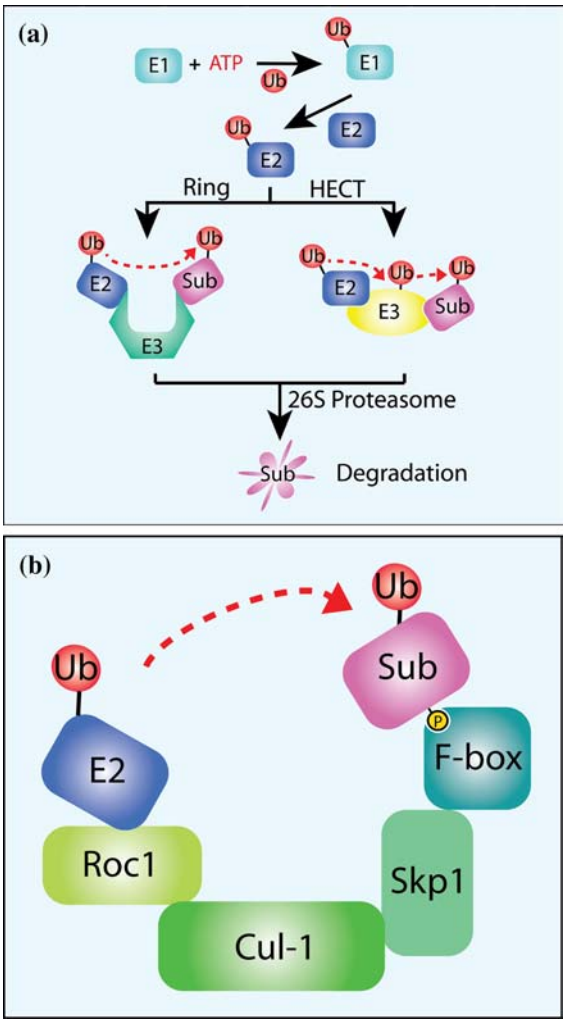

Figure 1. Ubiquitin (Ub)-mediated proteasomal degradation. (a) Proteins are targeted for degradation by the 26S proteasome through the covalent attachment of ubiquitin (Ub) moieties. The ubiquitination process is mediated by three consecutive enzymatic reactions. E1 activating enzyme utilizes ATP to form a high energy thioester bond with Ub. E2 conjugating enzyme accepts the Ub from E1, and conjugates Ub onto itself. Two types of E3 ligases are responsible for transferring the Ub from Ub-conjugated E2 to the targeted substrates. The HECT-domain E3 ligases transfer the Ub by forming a thioester bond between the E3 ligases and the Ub. Ub is then transferred again to the substrates. The RING-domain E3 ligases interact with the Ub-conjugated E2 which transfers the Ub directly to the substrates. (b) The schematic presentation of a major type of the RING-domain E3 ligases, SCF (*S*kp1–*C*ullin–*F*-box) complex. Components of SCF complexes include the scaffold protein Cullin-1, which interacts with SkpA and the F-box proteins at the amino-terminus. The carboxyl-terminus of Cullin-1 associates with the RING-domain interacting molecule Roc1, which in turn associates with Ub-conjugated E2 enzymes. Substrates are recognized through the carboxyl-terminus of F-box proteins and Ub is transferred to the substrates from E2. The interaction between the F-box proteins and the substrates requires the phosphorylation of substrates.

domain E3s act slightly different. The Ub is transferred first to the HECT E3s, and targeted to the substrates next. One of the best characterized RING ligases, the SCF (*S*kp–*C*ullin–*F*-box) complex, is composed of four major components: *S*kp1, *C*ul1/Cdc53, Roc1/Rbx1/Hrt1, and an *F*-box protein [Figure 1b, 4, 5]. Within the complex, Cul1 is the scaffold protein that interacts with Roc1 at its carboxyl-terminus to recruit Ub-conjugated E2. The amino-terminus of Cul1 interacts with Skp1, which binds to the F-box motif of an F-box protein. This F-box motif is approximately 50 amino acids long located at the amino-terminus and was named after the presence of this motif in mammalian cyclin F protein [5, 10, 11]. The carboxyl-terminal part of the F-box proteins usually consists of substrate-binding domains of various types, such as Trp-Asp (WD) repeats and leucine-rich repeats (LRR), both of which have been demonstrated to bind to phosphorylated substrates [12–14]. Depending on the motifs at the carboxyl-terminus, F-box proteins are categorized into three major families: the FBW family contains F-box proteins with WD repeats whereas those from the FBL family contain LRRs. The third family, FBX, consists of F-box proteins with other protein–protein interaction domains at the carboxyl-terminus such as carbohydrate-interacting (CASH), zinc-finger, and proline-rich domains [5, 10, 11].

The importance of F-box proteins are being dictated in several ways. First of all, F-box proteins are responsible for the substrate specificity of SCF complexes. F-box proteins connect the ubiquitination machinery and diverse cellular processes by exerting control over the stability of substrate proteins. In the mammalian system, the F-box proteins Skp2, β-Trcp and Fbw7 target substrates that control cell proliferation. Skp2 is responsible for degrading p27, an important regulator for S phase entry and other phases of the cell cycle [15–17]. Another F-box protein Fbw7 controls cell cycle progression by mediating the degradation of the G1 cyclins Cyclin E [18, 19]. Besides acting as a component of SCF complexes, F-box proteins have also been found to function in other biochemical contexts. For example, the

F-box protein, FOG-2, is required for spermatogenesis in *C. elegans* hermaphrodites [20]. FOG-2 binds to the RNA-binding protein GLD-1 through its FTH/DUF38 domain. This FOG-2/GLD-1 complex then in turn binds the 3′ untranslated region of *tra-2* mRNA in the germline cells and inhibits its translation [20, 21]. This inhibition is required for spermatogenesis to occur. In a quick glance, F-box proteins function in many different biochemical contexts and are involved in DNA replication, transcription, cell differentiation and cell death. As mentioned above, one of the most important functions that F-box proteins possess is to provide substrate specificity for the E3 RING ligases, SCF complexes.

F-box proteins are evolutionally conserved among eukaryotes like *H. sapiens*, *S. cerevisiae*, *C. elegans* and *D. melanogaster*. With powerful genetic techniques and explicit developmental tools, the modeling system *Drosophila* provides a well-established system for studying the F-box proteins. Currently, there are 33 F-box proteins encoded by the genome [4]. Table 1 lists all the *Drosophila* F-box genes with the proposed mammalian homologs and substrates for the reported F-box proteins indicated. Some of the F-box proteins are well characterized, and they have been shown to be involved in a number of developmental and signaling pathways. For example, one of the best characterized *Drosophila* F-box genes, *supernumerary limbs* (*slimb*), is the homolog of human F-box gene *β-Trcp* and controls the stability of several important signaling factors through SCF complexes. Among the substrates, Cubitus interruptus (Ci) and Armadillo (Arm, the human counterpart β-catenin) are the two downstream effectors controlling Hedgehog (Hh) and Wingless (Wg) signaling pathways, respectively [24, 31–33]. In the Dorsal/NFκB pathway, SCF^{Slimb} is responsible for the degradation of Cactus/IκB [25]. In addition, Slimb is involved in the regulation of circadian rhythm in flies [26, 27], plays multiple roles in *Drosophila* egg chamber development [34], and regulates the appearance of additional centrosomes during mitosis [35]. Another well-studied F-box gene, *archipelago* (*ago*), is implicated in the cell proliferation process. As a homolog of the mammalian gene *Fbw7*, Ago regulates the cell cycle positive regulator Cyclin E [22] and the growth controller dMyc [23] in *Drosophila*. Ago was also found to regulate the activity of Notch signaling pathway [36]. Evidently, the diversity in substrates implies a wide-range of cellular processes F-box proteins could potentially regulate. In this review, we focus on the functions of F-box proteins within the SCF complexes and their related molecular pathways in the modeling system *Drosophila*. Functions and molecular identities of three F-box proteins: Slimb, Ago, and Morgue, will be introduced and described in details below.

Table 1. Drosophila F-box genes and their substrates[a].

F-box families and their members	Reported F-box proteins	Mammalian homologs	Proposed substrates
FBW (3) *ago*, *slimb*, *CG9144*	Ago	FBXW7[b] [11]	CycE [22], dMyc [23]
	Slimb	FBXW1[b] (β-Trcp) [11]	Arm [24], Ci [24], Cactus [25], Period [26, 27]
FBL (11) *ppa, CG14891, CG7148, CG12402, CG13213, CG14937, CG11033, CG8873, CG8272, CG9003, CG1839*	Ppa	FBXL14[b] [11]	Paired [28]
FBX (19) *morgue, CG9461, CG11044, CG9772, CG9316, CG2247, CG4643, CG13766, CG6758, CG12765, CG13770, CG5003, CG7707, CG10855, CG4911, CG3428, CG2010, CG11866, CG5961*	Morgue		DIAP1[29, 30]

[a]Abbreviations: FBW, F-box proteins with WD motifs; FBL, F-box proteins with leucine-rich repeats; FBX, F-box proteins with other motifs or without recognizable motifs; CycE, cyclin E; Arm, Armadillo; Ci, Cubitus interruptus; Ago, Archipelago.
[b]Nonmenclature of mammalian F-box proteins is used according to Ref. [11].

Slimb regulates tissue growth and patterning

Growth and patterning during animal development rely on various signaling pathways. One such signaling pathway that directs proper transcription for genes required for development is the Hedgehog (Hh) pathway. The Hh pathway is largely conserved in *Drosophila* and vertebrates, with distinct features differed by evolutional divergence [31–33]. Figure 2a provides a brief overview of the Hh pathway in *Drosophila*. As a downstream effector of Hh, Ci mediates the transcriptional responses signaled by the Hh ligand. Ci is present in two major forms, the transcriptional repressor (CiR) and the transcriptional activator (CiA). The presence of CiA or CiR activates or represses the Hh-targeted gene transcription, respectively. The activity of Ci depends on the concentration of the Hh ligand perceived by the cell. In the absence of the Hh ligand, the 12-transmembrane receptor Patched (Ptc) inhibits the activity of a second transmembrane protein known as Smoothened (Smo). A protein complex which includes protein kinase A (PKA), casein kinase I (CKIα), glycogen synthase kinase-3β (GSK3β), a serine/threonine kinase Fused (Fu) and the scaffold kinesin Costal-2 (Cos2) is responsible for the phosphorylation of Ci when Hh is not present. Phosphorylated Ci then becomes exposed to the SCF^{Slimb} complex for ubiquitination. The SCF^{Slimb} complex mediates the processing of Ci and proteolyzes Ci into an amino-terminus fragment, CiR, the repressor form of Ci. CiR then translocates into the nucleus and represses the Hh-directed gene transcription. Upon the Hh ligand binding to the receptor Ptc, Smo is released from its inhibition, and becomes active through phosphorylation mediated by PKA, GSK3β, and CKIα. The group of kinases dissociates and the phosphorylation of Ci is ceased. The full length Ci is retained by the Suppressor of Fused (Sufu) in the cytoplasm, protected from proteolysis mediated by the SCF^{Slimb} complex, and moves into the nucleus to acts as an activator form, CiA, for directing Hh downstream gene transcription by interacting with the transcriptional co-activator CREB-binding protein (CBP).

The production of CiR is regulated by the F-box protein Slimb and its associated degradation machinery. Slimb was first discovered from a genetic screen for recessive mutations that alter normal adult patterning in *Drosophila* [24]. Three alleles of *slimb* were identified and with the stronger alleles, clones of mutant cells generated by mitotic recombination were found to exhibit phenotypes that recapitulate the ones induced by ectopic activation of the Hh and Wg pathways [24]. During normal limb development, Hh is secreted from the posterior compartment to induce the expression of either Decapentaplegic (Dpp) or Wg in anteriorly adjacent cells. Activities of these morphgens control tissue patterning in both posterior and anterior compartments. Interestingly, while posterior mutant clones of *slimb* develop normally, anterior ones ectopically express Dpp or Wg. These phenotypes are similar to the ones caused by clones of cells expressing Hh ectopically. Furthermore, full length Ci predominantly accumulates in the *slimb* mutant cells as it does in the *PKA* mutant cells and cells responsive to Hh, suggesting that Ci is protected from proteolysis mediated by Slimb and PKA activities in the presence of Hh signaling [24].

Like Hh, the Wg signaling pathway plays a crucial role in *Drosophila* wing development. Wg expression is induced in a narrow strip of cells along the dorsoventral compartment boundary [37–39]. The Wg protein is secreted into neighboring cells and the signal is transduced through the ligand–receptor interaction between Wg and members of the Frizzled seven transmembrane-domain receptor family (Figure 2b). The binding of Wg to the Frizzled receptors emanates the phosphorylation of downstream target Dishevelled (Dvl). Phosphorylated Dvl inhibits the activity of protein complex composed of the scaffold Axin, Zeste-White-3 (GSK3β) and Adenomatous Polyposis Coli (APC) [40]. As the Axin protein complex is disabled by the phosphorylated Dvl, phosphorylation of Armadillo (Arm), the downstream effector of the Wg pathway, ceases and Arm is protected from proteolysis mediated by the SCF^{Slimb} complex. The Arm protein is therefore stabilized and available for directing Wg downstream gene transcription [40, 41]. *Vice versa*, when the Wg ligand is absent, phosphorylation of Dvl is not induced and the Axin protein complex is active. Phosphorylation by the Axin protein complex marks Arm and exposes it to the SCF^{Slimb} ubiquitination machinery. Notably, the Arm protein is accumulated cell autonomously in the mutant clones of *slimb* in *Drosophila* wing discs

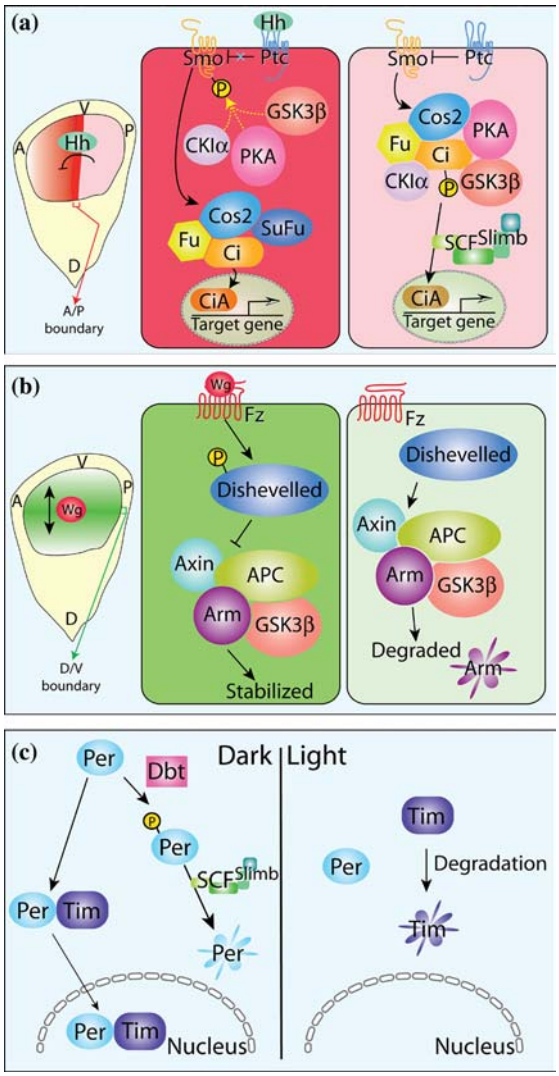

Figure 2. The *Drosophila* F-box protein Slimb regulates major developmental pathways. (a) Slimb targets Cubitus interruptus (Ci) and controls its stability by proteolyzing the full length Ci (CiA) into CiR, a carboxyl-terminus truncated repressor. In *Drosophila* wing discs, the Hedgehog (Hh) ligand is secreted from the posterior to the anterior compartment. In the presence of Hh ligand, the activity of receptor Patched (Ptc) is inhibited, leading to the activation of the transmembrane receptor Smoothened (Smo) that also depends on the phosphorylation by kinases GSK3*beta*, CKI*alpha*, and PKA. Ci then reassociates with Sufu (Suppressor of Fu) and remains in its full length activated form CiA. CiA translocates into the nucleus and activates Hh-directed gene transcription. On the contrary, when the Hh ligand is absent, Ptc is active and inhibits the activity of Smo. Ci is phosphorylated by the kinases, targeted by the SCF^{Slimb} complex, and processed into the CiR fragment which acts as a repressor to shut down the Hh-directed gene transcription. (b) Slimb is involved in the degradation of Armadillo (Arm), a downstream effector for the Wingless (Wg) pathway. The Wg ligand interacts with the receptor Frizzled to enable the phosphorylation of Dishevelled (Dvl). Once Dvl is phosphorylated, it inhibits the activity of Axin and stabilizes the Arm protein. On the other hand, the absence of Wg inactivates Dvl. The Axin-containing complex is active and phosphorylates Arm due to the inactivation of Dvl. Upon phosphorylation, Arm becomes a target for the SCF^{Slimb} complex and is degraded. (c) Slimb is a key player in the mechanism of *Drosophila* circadian rhythm. During darkness, Period (Per) interacts with Timeless (Tim) in its unphosphorylated state and translocates into the nucleus as Per–Tim heterodimers. When Per does not associate with Tim, it undergoes phosphorylation by Double-time (Dbt) and is targeted for degradation by the SCF^{Slimb} complex. In the light, nevertheless, Tim is targeted for rapid degradation.

[24]. The accumulation of Arm protein in *slimb* mutant clones suggests that Slimb is involved in destabilizing Arm in the absence of Wg signaling. Similar to the Hh pathway, the absence of Slimb protects Arm from proteolysis event, mimicking what occurs upon the activation of the Wg signaling pathway [24].

Slimb as a biological clock indicator

A third substrate for the F-box protein Slimb is the Period (Per) protein. In *Drosophila*, circadian rhythms are controlled through the interaction between the Per and Timeless (Tim) proteins [42, 43]. Oscillations in level and phosphorylation status of these two proteins profile the animal body clock. The oscillations pace is well regulated during the darkness whereas the daylight disrupts the regular oscillation cycle. In constant darkness, Per and Tim associate physically. Their mRNA levels cycle in the same phase and period, as their protein products Per and Tim. These two proteins form heterodimers and negatively regulate their own gene transcription. The oscillation pace continues, until the animal is exposed to daylight, which results in the rapid degradation of Tim [43]. The physical interaction between Per and Tim also translocates the heterodimer into the nucleus, whereas individual proteins prior to the assembly are retained in the cytoplasm. This heterodimeric assembly, that establishes the criteria for nucleus entry, serves as a checkpoint for the circadian cycle and is required for the proper rhythmicity to proceed [44].

Reports suggest that oscillations occur not only in the levels of *per* and *tim* mRNAs and Per and Tim proteins but also in regards to the phosphorylation of two proteins. A third clock gene, *double-time* (*dbt*), was isolated from a genetic screen for locomotor rhythm mutations [45, 46]. *dbt* gene encodes a protein closely related to human casein kinase Iε, and plays a prominent role in regulating the phosphorylation and abundance of Per protein. Using cell culture system, researchers have mimicked the features observed in flies for the regulation of Per by Dbt [26, 35]. Transfection analyses have showed that Dbt promotes the progressive phosphorylation of Per, and that hyperphosphorylated Per is rapidly degraded by the Ub-proteasome pathway. Several lines of evidence indicate that the F-box protein, Slimb, is the key player mediating the degradation of Dbt-phosphorylated Per. Wild-type Slimb interacts preferentially with the phosphorylated form of Per whereas a mutant form of Slimb that lacks the F-box motif leads to the accumulation of phosphorylated Per in the presence of Dbt, suggesting that the F-box motif (i.e. interaction with the SCF complex) is required for the degradation of phosphorylated Per. Moreover, gene silencing of *slimb* using RNAi strategy in cell culture system efficiently blocks the degradation of Per, further reinforcing the conclusion that Slimb is responsible for mediating the destruction of Per protein. Genetically, flies overexpressing *slimb* or the dominant-negative version of *slimb* exhibit arrhythmic clock activities [26, 35]. While in constant darkness, highly phosphorylated forms of Per and Tim are present at high levels when Slimb is absent, indicating that normal cyclic degradation is impaired.

Archipelago of destruction

Central to the cell developmental mechanism is the process of the cell cycle, a basic cyclic progression formed by repetitive events including the S phase for the replication of DNA and the M phase for initiation of genome segregation. Cells grow and divide through checkpoints at each stage of the cell cycle while correct timing is under the control of cyclin proteins and the Ser/Thr protein kinases known as cyclin-dependent kinases (CDKs) [47, 48]. Studies on the regulation of cell cycle progression have suggested that excess activities of CDK2-Cyclin E complexes cause premature entry into S phase and development of human tumors, implicating an important role for Cyclin E in cell cycle regulation. Previous literature has indicated that the level of Cyclin E is modulated by degradation events [49, 50]. Hariharan and the coworkers decided to establish a genetic screen in *Drosophila* which aims to identify genes involved in controlling cell numbers and growth during development [22]. The screen was done by generating clones of homozygous mutant tissue in fly eyes and selecting the mutant cells that had a proliferative advantage over the wild type cells. Homologs of human tumor repressor genes such as *PTEN* were identified in this screen, directly validating this approach. A group of the isolated fly strains carried mutations on the gene *archipelago* (*ago*). Within the *ago* mutant clones, distance between adjacent photoreceptor clusters was increased compared with the wild-type. Within the enlarged space of *ago* mutant clones are excess cells, indicating overproliferation in the mutant tissue. Overproliferation of *ago* mutant tissue is not due to irregular apoptosis event since there is no significant decrease in the extent of cell death [22]. Taken all the results together, *ago* is believed to control cell proliferation with this function regulated by apoptosis.

ago encodes a 1326-amino acid protein which contains an F-box motif at the amino-terminus and seven WD repeats at the carboxyl-terminus. Due to its role in cell proliferation, researchers suspected that loss of *ago* could lead to an increased level of a positive cell cycle regulator. Several cyclins and CDKs were examined, with the G1–S cyclin Cyclin E found to accumulate in the *ago* mutant clones in third instar eye imaginal discs [22]. Nevertheless, the mRNA level of *cyclin E* remains unchanged within *ago* mutant clones, suggesting a post-transcriptional modification on Cyclin E by Ago. Furthermore, a smaller fraction of G1 phase cells was found in the *ago* mutant cells compared with the wild type cells by flow cytometry. Likewise, the proportion of cells found in S and G2/M phases was also higher in the mutant cells than the wild type. These results suggested that the increased protein level of Cyclin E in *ago* mutant cells disrupts exit from the cell cycle (thus resulting in continued proliferation) in a manner

similar to that evoked by the overexpression of Cyclin E. In addition, 3 out of 10 ovarian cancer cell lines were examined and found to have mutations in the human *Fbw7* gene, the homolog of *ago*. Cyclin E is upregulated in these ovarian cancer cell lines as well, implicating a role of *ago* in human cancer pathogenesis [22].

A feature observed in *ago* mutant cells, the increase in cell growth, is not observed in the cells overexpressing Cyclin E. This inconsistency prompts Moberg and the colleagues to conduct two different interaction screens using the Ago F box/WD domain as a bait to search for additional substrates of Ago [23]. A *Drosophila* ortholog of the Myc transcription factor, dMyc, was recovered from these screens. Myc genes encode basic-helix–loop–helix–zipper (bHLHZ) domain transcription factors and dimerize with Max family proteins through the bHLHZ domain to promote cell growth and proliferation [51, 52]. The Max/Myc heterodimers bind sequence specific DNA-like E box binding sites and modulate transcription activities. In *Drosophila*, overexpression of dMyc causes cell over growth as evidenced by the increased size of developing tissues [53, 54]. This phenotype is consistent with *ago* mutant tissues. While both dMyc and Cyclin E promote S phase entry, only dMyc was found to have effect on growth [53, 54]. Mutant *dMyc* flies are smaller than the wild-type flies in body size, and losing a copy of wild-type *ago* gene under the *dMyc* mutant background restores the body size defect to the extent of 12 to 15% [23]. Similar to Cyclin E, protein levels of dMyc were also elevated in *ago* mutant clones and its mRNA level remains the same. Direct interaction between dMyc and Ago were also observed by cell transfection and coimmunoprecipitation analyses [23]. To summarize, Ago regulates the degradation of both Cyclin E and dMyc, and the increased cell growth observed in tissues ectopically expressing dMyc mainly accounts for the *ago* mutant phenotype; overexpression of Cyclin E does not have an effect on cell growth, nevertheless, it is regulated by Ago in the aspect of promoting S phase entry and cell cycle progression (Figure 3).

Morgue's cell death program

Upon division, individual cell struggles to survive. Cells also face the choice of life and death, the decision of which is made via various mechanisms and regulators. Scientific findings have suggested that cell death is programmed and under the control of complex regulatory networks [55]. A group of cysteine proteases named caspases have been found to degrade or process various structural and enzymatic proteins during the dismantling of a cell. Caspases are activated by proteolysis events which generate mature heterotetramers upon cleavage of the pro-caspases [55–57]. The activities of caspases are inhibitied by a group of proteins called Inhibitor-of-Apoptosis Proteins (IAPs) [58, 59, 60]. IAPs contain a BIR (Baculovirus IAP Repeat) domain that interacts with caspases and a RING domain for the ubiquitination of capases. The caspase activity is blocked and programmed cell death is repressed once the caspases are bound to the IAPs and

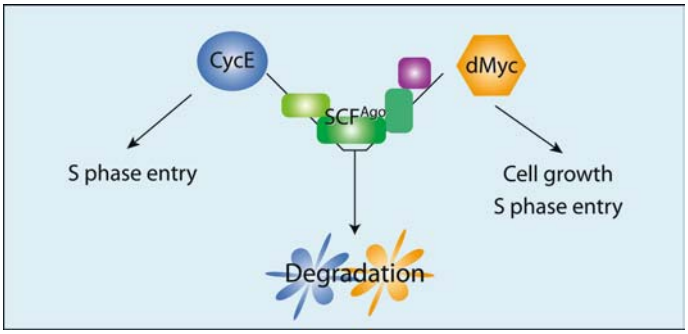

Figure 3. The *Drosophila* F-box protein Archipelago (Ago) regulates cell proliferation. The representative diagram indicates that SCF^{Ago} controls the cell cycle through two substrates: CycE and dMyc. Ago mediates the degradation of CycE and prevents the excessive accumulation of CycE protein at the G1–S boundary of cell cycle to ensure the proper entry of S phase. In addition, Ago also degrades dMyc, a regulator of cell growth and S phase entry.

ubiquitinated. One of such IAPs is *diap1* in *Drosophila*, where loss of the *diap1* gene results in massive, ectopic programmed cell death [61]. In contrast, loss of the "*grim-reaper*" genes in *Drosophila* results in the blockade of cell death. Ectopic expression of the "*grim-reaper*" complex induces cells to die [62–64]. These genes promote cell death by protein–protein interaction with DIAP1, thereby blocking the caspases from interacting with DIAP1. As a consequence, caspases become active in dying cells. A double-inhibition model of programmed cell death has been proposed, which centers on three major players: Caspases, DIAP1, and Grim-Reaper (Figure 4a). In the cells that survive, DIAP1 binds and inhibits caspases. Caspases are also targeted for ubiquitination through interaction with DIAP1 in the surviving cells. On the other hand, cells are promoted for death when Grim-Reaper displaces caspases to interact with DIAP1 instead. The free caspases are activated by proteolysis and further promote cell death in the dying cells [65].

The Ub-proteasome degradation pathway is implicated in programmed cell death by the *Drosophila* F-box gene, *morgue* (Figure 4b). Cell death modifier screens were performed by two independent laboratories and *morgue* was isolated as an enhancer of the phenotype caused by overexpression of *grim-reaper* using *GMR-gal4* in *Drosophila* eye imaginal discs [29, 30]. During normal eye development, one-third of the lattice precursor cells are eliminated by apoptosis, ensuring that a refined hexagonal interommatidial lattice is formed in the mature retina [29, 30]. Mutations in *morgue*, however, impair apoptosis in the developing retina as evidenced by the formation of extra cells in the pigment cell lattice. Morgue is predicted to be a 491-amino acid protein and carries an F-box domain and an Ub conjugase domain. Due to the presence of the Ub conjugase domain in Morgue, the function of Morgue is more diverse than the regular WD motif containing F-box proteins. It is possible that Morgue acts as a regular F-box protein by working in conjunction with SkpA (the fly homolog of Skp1) in a specific SCF E3 ligase complex to mediate the ubiquitination of DIAP1. The Ub conjugase domain that Morgue possesses differs in a glycine residue from the cysteine required for Ub attachment. Therefore, the conjugase domain is a variant of the regular E2 Ub conjugase domain and is rendered inactive due to the lack of the catalytic cysteine residue. Morgue may require this conjugase domain to recruit the RING domain substrates onto the Morgue-containing SCF complex and target the substrates for ubiquitination. This conjugase domain may also act as an Ub enzyme E2 conjugase variant (UEV) which, despite its inactive status, may function along with a catalytically active E2 conjugase to promote protein ubiquitination. These results suggest Morgue provides an additional level of regulation on caspases in living cells. Morgue modulates the protein turnover of DIAP1 by targeting DIAP1 for ubiquitination and degradation, providing another example of F-box proteins implicated in the regulation of diverse cellular machineries.

Concluding remarks

Among all the complex mechanisms that animal organisms utilize to maintain life and survival, $SCF^{F\text{-}box}$-mediated ubiquitination processes provide control over the stability of proteins and play essential roles in many aspects of development. In this review, three important F-box proteins are discussed on how they are involved in different signaling pathways through their ability to target specific proteins for degradation, providing some insight into how exactly F-box proteins perform their actions. These examples are only a part of the whole range of F-box-dependent processes that will continue to expand. Over the past decades, researchers have continuously put in effort to unravel the functions of F-box proteins and more questions are raised thereafter. For example, F-box proteins are numerous and diverse in functions, are there overlaps among different F-box proteins in choosing proteins to be targeted for degradation? If there is redundancy in utilizing two or more F-box proteins to target the same substrate, how and why organisms decide to use such strategy to exert control over the stability of the substrate? In addition, how many substrates does an F-box protein possibly possess? Are there any connections between individual substrates for the same F-box protein? How exactly an F-box protein chooses the substrates besides the

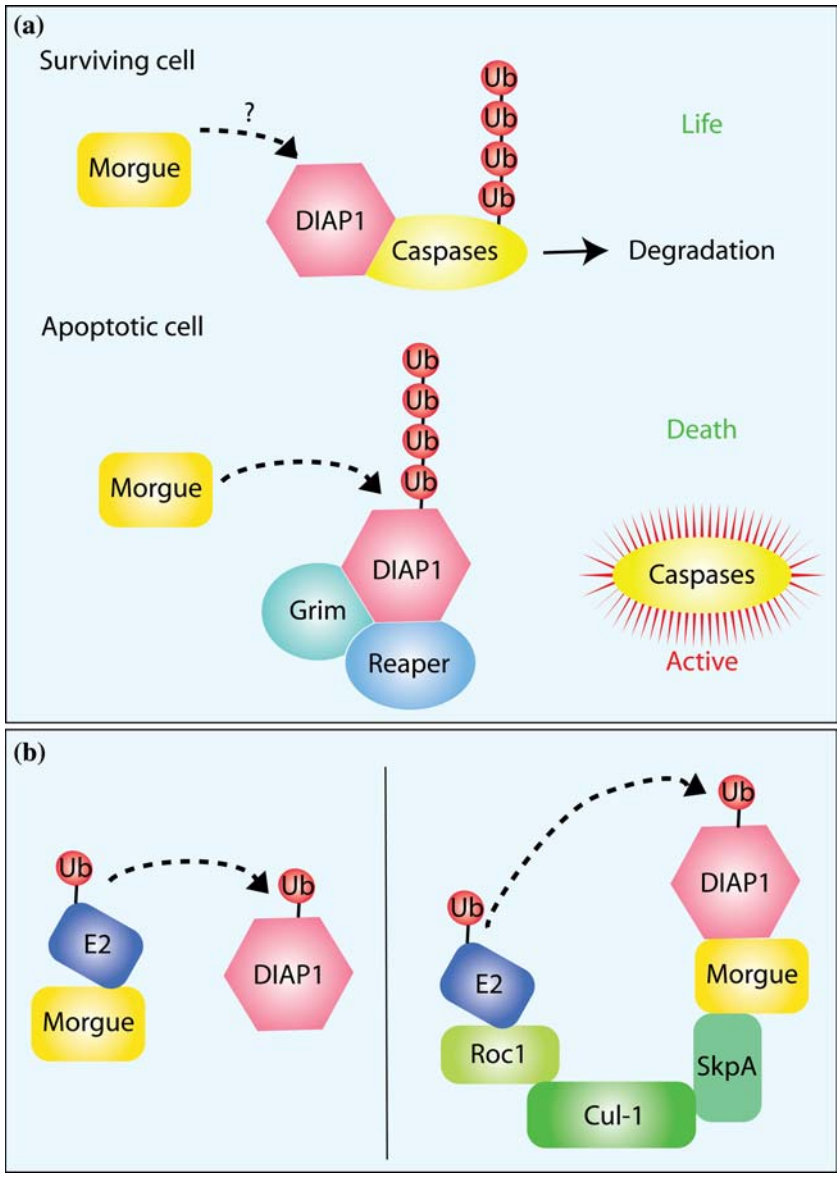

Figure 4. Morgue is an F-box protein that controls the cell death. (a) Programmed cell death is controlled by three major players: DIAP1, caspases, and Grim-Reaper. Caspases interact and are degraded by DIAP1 in surviving cells through the ubiquitination activity of DIAP1. In the dying cells, however, Grim-Reaper interacts with DIAP1 and displaces caspases. Thus, free caspases are available for promoting cell death. In both cases Morgue provides an additional level of control on cell death by regulating DIAP1 protein turnover. (b) The F-box protein Morgue regulates the degradation of DIAP1 via two possible mechanisms. Morgue may interact with functional E2 conjugating enzyme through its conjugase domain and ubiquitinate DIAP1 (left panel). Alternatively, Morgue functions as a regular F-box component within the SCF complex to provide the substrate specificity and degrade DIAP1 in this route (right panel).

commonly required phosphorylation status of the targeted protein? Answers to these questions will ultimately elaborate the functional properties of F-box proteins, further enhancing our knowledge on the regulation of life and death of proteins and cells.

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