The Fbw7 and Beta-TRCP E3 ubiquitin ligases and their roles in tumorigenesis

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1. ABSTRACT

The Ubiquitin Proteasome System (UPS) is a major regulator of protein abundance in the cell. The UPS influences the functions of multiple biological processes by targeting key regulators for destruction. E3 ubiquitin ligases are a vital component of the UPS machinery, working with E1 and E2 enzymes to bind substrates and facilitate the transfer of ubiquitin molecules onto the target protein. This poly-ubiquitination, in turn, directs the modified proteins for proteolysis by the 26S proteasome. As the UPS regulates the degradation of multiple oncogenes and tumor suppressors, the dysregulation of this pathway is known to promote various diseases including cancer. While E1 and E2 enzymes have only been minimally linked to cancer development, burgeoning amounts of evidence have implicated loss or gain of E3 function as a key factor in cancer initiation and progression. This review will examine the literature on two SCF-type E3 ligases, SCF^{Fbw7} and SCF$^{beta-TRCP}$. In particular, we will highlight novel substrates recently identified for these two E3 ligases, and further discuss how UPS regulation of these targets may promote carcinogenesis.

2. INTRODUCTION

Cellular proteins are often in a constant state of flux. This dynamic state of protein expression is important for numerous signaling and cell process controllers, as these proteins must only be present when needed and quickly degraded. Regulators that are present within a cellular system for too long or too short can drastically affect the signaling pathway that they mediate (1). Therefore, the biological route that sustains the proper temporal regulation of these proteins is of critical importance. The Ubiquitin Proteasome System (UPS) is one of the key pathways that execute this function within the cell (1). The UPS is a well-organized destruction machine with multiple protein components (ubiquitin-activating E1 enzymes, ubiquitin-conjugating E2 enzymes, ubiquitin-protein E3 ligases, and the 26S proteasome) working in concert with one another to ensure the timely and efficient proteolysis of target substrates. As such, dysregulation of any component of the UPS can significantly affect the output of any given biological process under its regulation.
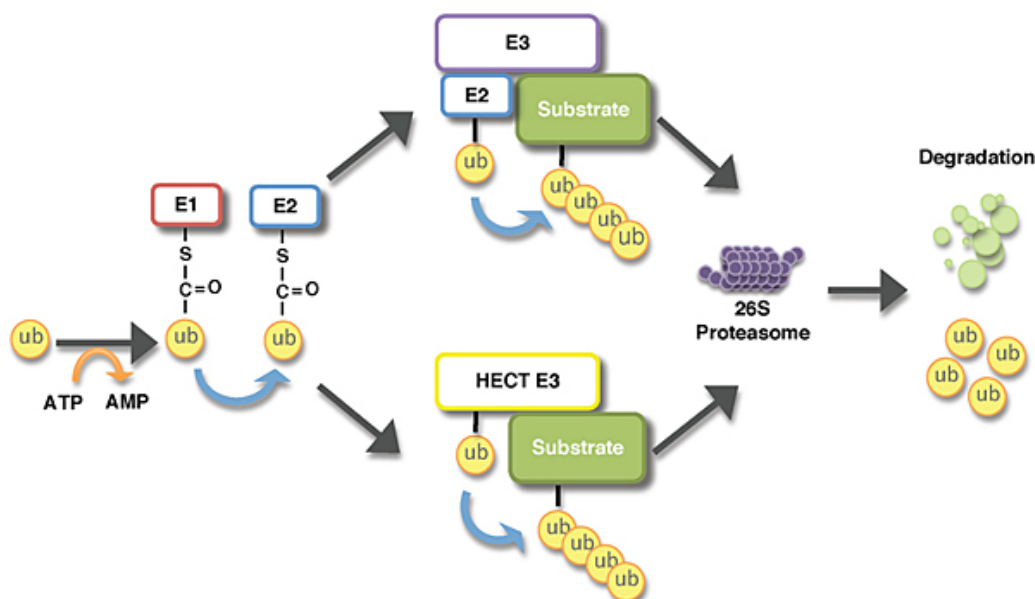
Figure 1. Illustration of the Ubiquitination Reaction Pathways. Initially, the E1 enzyme activates, in an ATP-dependent manner, the 76 amino acid ubiquitin molecule by forming a high-energy thiol ester bond with ubiquitin. This activated molecule is then transferred to the E2 conjugating enzyme. The E3 ligase then positions the target substrate near the E2 enzyme allowing for the transfer of ubiquitin. In the case of HECT E3 ligases however, the E3 ligase directly transfers the ubiquitin molecule onto the target substrate. Once a chain of four or more ubiquitin molecules is placed onto the substrate protein, the molecule is then targeted for proteolysis by the 26 proteasome.

3. THE UPS

The degradation of proteins by the UPS is mainly comprised of two steps: 1) the covalent attachment of multiple ubiquitin moieties to the protein substrate and 2) the degradation of the poly-ubiquitinated protein by the 26S proteasome (Figure 1). The first step in this regulatory pathway requires the concerted efforts of three enzymes: an ubiquitin-activating E1 enzyme, an ubiquitin-conjugating E2 enzyme, and an ubiquitin-protein E3 ligase. The E1 enzyme activates, in an ATP-dependent manner, the 76 amino acid ubiquitin molecule by forming a high-energy thiol ester bond with ubiquitin. Next, the activated ubiquitin molecule is transferred to the E2 enzyme, which facilitates the binding of the E3 ligase. Each E3 ligase positions the target protein near the E2 molecule allowing for the transfer and covalent bonding of the ubiquitin moiety (2). It should be noted that this mechanism of conjugation is not specific for all families of E3 ligases. The human genome encodes several types of E3 ligases that are mainly characterized by the presence of either a HECT (homologous to E6-associated protein C-terminus), RING (really interesting new gene) or U-box domain (3). While RING and U-box E3 ligases act as a scaffolding protein that bring substrates and E2 enzymes together, HECT E3 ligases can directly catalyze the conjugation of the ubiquitin molecule onto the target protein (Figure 1).

The ubiquitination of the substrate occurs between the carboxy-terminus of ubiquitin and a lysine epsilon-amino group on the target protein. One ubiquitin molecule placed on a target substrate is defined as mono-ubiquitination. This type of post-translational modification influences various cellular processes including growth factor-induced endocytosis, DNA repair, and chromatin modification/transcriptional regulation (4). In addition, ubiquitin has several acceptor lysines to allow for further ubiquitin conjugation. This flexibility permits for additional changes in the length of the modification and consequently further alteration in the function of the modification. While ubiquitin has seven lysine residues that may participate in chain elongation, K48 and K63 are the most thoroughly characterized acceptors thus far. Poly-ubiquitin chains of four or more K48-linked moieties mainly function as a signal for proteasome-mediated degradation (5). On the other hand, K63-linked ubiquitin chains regulate a wide-range of non-proteolytic cellular processes including DNA repair, viral resistance and kinase activation (6). While the functions of the other ubiquitin-linked chains are not as well studied, some intriguing observations have been made concerning their physiological roles within the cell. For instance, BRCA1:BARD1-mediated K6-linked ubiquitin chains have been implicated in DNA replication and repair (7). K11-linked chains are preferentially employed for endoplasmic reticulum-associated degradation (ERAD) (8), Tumor Necrosis Factor (TNF) signaling (9), and Anaphase-Promoting Complex (APC)-mediated proteolysis (10). Additionally, K27- and K33-linkages are utilized during the stress response (11), and K29-linked chains play a prominent role in ubiquitin fusion protein degradation (12) and lysosomal degradation (13).

Similar to phosphorylation (14) and sumoylation (15), ubiquitination is also a reversible post-translational

modification that is subjected to bi-directional regulation. The removal of the ubiquitin moiety is mediated by a group of de-ubiquitinating enzymes (DUBs) that can be subdivided into five families: ubiquitin C-terminal hydrolases (UCHs), ubiquitin-specific proteases (USPs), ovarian tumor-like proteases (OTUs), JAMM/MPN+ metalloproteases, and the Josephins (16). DUBs have multiple functions within the UPS. First, they are capable of cleaving ubiquitin chains from modified substrates, rescuing the protein from proteasomal degradation. Second, the removal of ubiquitin by DUBs can also effectively end any non-proteolytic function that the modification may have provided. Finally, these enzymes also perform a "house cleaning" role by cleaving ubiquitin chains from proteins entering the 26S proteasome (17). This house cleaning function recycles ubiquitin molecules allowing for their further use in this degradation pathway. Therefore, DUBs play a vital role in the UPS. They insure a continuous pool of usable ubiquitin while further providing a layer of regulation for this prominent cellular pathway.

4. THE UPS AND CANCER

Tumorigenesis is often the outcome of compounding genetic mutations in multiple biological processes including those that regulate genomic stability, cell proliferation, DNA repair, cellular differentiation, and motility. Short-lived proteins whose abundance is mediated by the UPS frequently control these vital cell tasks. This type of temporal protein regulation is evident with the cyclins, transiently expressed proteins whose functions are important for proper cell cycle progression. Cyclin E, for instance, only accumulates at the G1/S boundary and facilitates entry and progression through the S-phase by activating cyclin-dependent kinase 2 (Cdk2) (18, 19). After performing its function, Cyclin E is quickly degraded by ubiquitin-mediated proteolysis (20). This process is essential as prolonged expression of Cyclin E can lead to genomic instability and polyploidy, which may ultimately result in disease (21).

Dysregulation of the UPS machinery has often been associated in the initiation and promotion of various pathophysiological conditions including cancer. While the concerted efforts of the E1 enzymes, E2 enzymes, and E3 ligases are crucial to the proper conjugation of the ubiquitin moiety, no reports have linked E1 enzymes to cancer and only a few studies have shown the role of E2 enzymes in tumorigenesis (22). However, a large amount of evidence demonstrates that the dysregulation of E3 ligases can promote the various stages of the transformed phenotype (23). As targets of various E3 ligases include known oncoproteins and tumor suppressors, the inactivation of a given E3 ligase may stabilize an oncogene substrate, or reciprocally, over-expression of a specific E3 ligase may enhance the proteolysis of a tumor suppressor protein (24). For example, over-expression of Murine double mutant 2 (Mdm2), which is the E3 ligase for the p53 tumor suppressor, has been linked to breast cancer. *In vivo* studies demonstrated that a polymorphism in Mdm2 could lead to a two-fold over-expression of the protein that was significant enough to reduce the function of p53. This tumor

suppression reduction subsequently led to a considerable increase in risk for hormone-dependent breast cancer in women (25, 26). In addition, the oncoprotein Akt kinase was recently reported to be regulated by a degradation mechanism involving the E3 ligase TTC3 (27). Moreover, cellular levels of the tumor suppressor phosphatase and tensin homolog (PTEN) are regulated by the HECT-type E3 ligase NEDD4-1 (28).

While there are multiple E3 ligases that have been implicated in the development and progression of cancer, this review will focus predominately on two well-characterized RING-E3 ubiquitin ligases that are members of the SKP1-CUL1-F-box-protein (SCF) complex family of ligases. A growing amount of evidence has been collected showing the dysregulation of this family of E3 ligases in carcinogenesis.

5. THE SCF-TYPE OF E3 UBIQUITIN LIGASES

The SCF family of ubiquitin E3 ligases was originally identified in *Saccharomyces cerevisiae* and catalyzed the phosphorylation-dependent ubiquitination of cell cycle machinery (29, 30). This group of enzymes is best characterized for its role in regulating the mammalian cell cycle, however, later research showed that its functions were not restricted to this process. Research by various groups have also placed these E3 ligases in multiple cellular pathways including apoptosis (31-33), cell differentiation (34, 35), lipid metabolism (35, 36), and development (37-39). Structurally, this family of proteins is composed of three static subunits and a variable subunit. The three static subunits include a catalytic RING subunit (Rbx1), a scaffolding subunit (Cul1), and an adaptor subunit (Skp1). The variable molecule is known as the F-box protein (FBP) (Figure 2**)**. The FBP is the substrate recruitment module of the E3 ligase complex. Therefore, the identity of the F-box protein determines the target of the SCF ligase. Furthermore, unlike HECT E3 ligases that can directly conjugate ubiquitin onto the target substrate, the structural organization of the SCF complex serves to bridge the interaction between the E2 enzyme and the target protein (Figure 2).

The term F-box was originally derived from Cyclin F, the first characterized protein containing the F-box motif. This motif is a 40 amino acid sequence that is required to bind Skp1 (30). To date, approximately 69 FBPs have been identified in the human genome, allowing this family of ligases to target a wide range of proteins. F-box proteins interact with substrates via its C-terminal protein binding domains, and can be further classified into three sub-families based on these domains. These categories include FBPs that contain WD40 repeats (FBXW), leucine rich repeats (FBXL) or other domains (FBXO) (Figure 3) (40). Unlike APC/C type ligases that recognize KEN Box or Destruction Box (D-box) sequences (41, 42), FBPs typically require an additional post-translational modification for discrimination. In most cases, phosphorylation of the F-box recognition motif present on the substrate, more commonly known as a phospho-degron, is necessary before efficient substrate/ligase interaction (3).
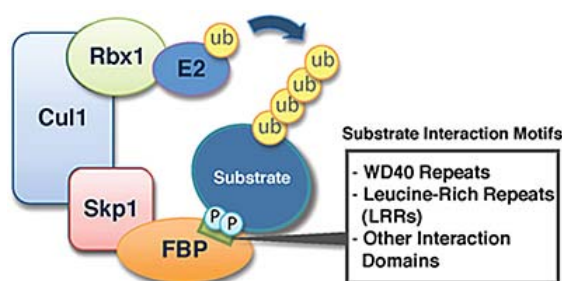
Figure 2. Structural Illustration of the SCF Family of E3 ligases. An SCF-type E3-ligase is a multi-subunit complex consisting of three invariable subunits and one variable subunit. The three static subunits include a catalytic RING subunit (Rbx1) that interacts with the E2, a scaffolding subunit (Cul1), and an adaptor subunit (Skp1). The variable molecule is known as the F-box protein (FBP). The major function of the F-box protein is to recruit specific substrates to the E3 complex via substrate interaction domains. The two largest classes of interaction domains found on FBPs are WD40 repeats and leucine-rich repeats (LRRs). A third type of FBP also exists which contains neither WD40 repeats nor LRRs. This third class of F-box proteins contains other types of interaction domains or no recognizable domain at all. To date, there have been approximately 69 FBPs identified in the human genome. Furthermore, unlike HECT E3 ligases that can directly conjugate ubiquitin onto the target, SCF complexes bridge the interaction between the E2 enzyme and the substrate.

The additional layer of regulation is important as it allows for added discretion when targeting short-lived regulatory molecules for proteolysis. This phosphorylation-dependent regulation has even greater depth, as multiple phosphorylation events are sometimes required. In the case of Mdm2 (43), Sic1p (44), and Gli3 (45), phosphorylation at several sites are necessary before their putative SCF enzyme can efficiently recognize the protein. In addition, other proteins may require sequential phosphorylation in which the first phosphorylation event "primes" a target protein for a secondary modification. A classical example of this type of regulation is seen with the c-Myc oncoprotein. For efficient degradation of c-Myc, a MAPK-dependent phosphorylation must first occur at Ser62, which primes the protein for a subsequent phosphorylation by GSK3-Beta at Ser58. Phosphorylated Ser58 then serves as the modified phospho-degron that is recognized by its E3 ligase (46, 47).

As F-box proteins play such a fundamental role in the proper functioning of SCF ligases, loss of function or mutations in this protein can ultimately lead to cancerous implications. For instance, one well-studied F-box protein, Fbw7, is a *bona fide* tumor suppressor, and work from multiple laboratories has implicated this FBP in various human cancers including cholangio-carcinoma and T-cell acute lymphocytic leukemia (T-ALL) (48, 49).

6. THE SCF[FBW7] E3 UBIQUITIN LIGASE

The first member of the *FBW7* gene family, Cdc4, was originally identified in a genetic screen for temperature sensitive cell division cycle (cdc) mutants in *Saccharomyces cerevisiae* (50). Since its discovery in yeast, orthologs of Cdc4 have been characterized in other species including *Caenorhabditis elegans* (SEL-10) (51), *Drosophila melanogaster* (Archipelago) (52), *Mus musculus* (53) and *Homo sapiens* (Fbw7, also known as Fbxw7) (20, 54). While substrates that are recognized by SCF ligases play roles in various biological processes, prominent SCF[Fbw7] targets include proteins important in cell division/growth and survival. Well-characterized substrates of this E3 ligase include Cyclin E (20, 52, 54), c-Myc (46, 47, 55), c-Jun (56, 57), Mcl-1 (32, 33), sterol regulatory element binding protein-1 (SREBP1) (58), mTOR (59) and Notch-1 (34, 60, 61). Structurally, Fbw7 contains several protein-protein interaction motifs. In addition to its F-box motif, this FBP possesses eight C-terminal WD-40 repeats that form a beta-propeller binding pocket. Present within this protein structure are three conserved arginine residues that form contacts with phosphorylated targets (62, 63). These targets typically contain the conserved Cdc4 phospho-degron (CPD) (L)-X-pT/pS-P-(P)-X-pS/pT/E/D (X = any amino acid) (24). Similar to other members of the SCF ligase family, the destruction motif must first be phosphorylated in order for Fbw7 to efficiently recognize and mediate the ubiquitination of the target protein. Interestingly, GSK3 kinase can instigate the degradation of several, if not all, targets of Fbw7. This further solidifies the role for Fbw7 in cell growth and survival as GSK3 is subject to inhibition by mitogen signaling through the PI3K-Akt pathway (64, 65).

Human *FBW7* encodes for three protein isoforms (alpha, beta, and gamma) derived from alternative splicing and resulting in protein products that only vary at the N-terminus (66). Each isoform is differentially regulated both in tissue expression and cellular locale. In mice, the alpha isoform is ubiquitously expressed in all tissues, while the beta isoform displays higher levels in the brain and the gamma isoform exhibits increased expression in muscle tissue (67). The localization of each isoform also varies within the cell and is located in distinct sub-cellular compartments. Fbw7-alpha is nucleoplasmic, Fbw7-beta is cytoplasmic, and Fbw7-gamma is nucleolar (68). While the exact physiological significance of this spatial regulation is still unclear, it may allow for a more precise and localized regulation of Fbw7 activity, perhaps even accounting for a certain level of substrate specificity for each isoform. Interestingly, several studies have begun to shed some light on isoform specific functions for Fbw7. One report demonstrated that Fbw7-gamma co-localizes with a subpopulation of c-Myc in the nucleolus and can regulate the nucleolar accumulation of c-Myc (68). Moreover, poly-ubiquitination and turnover of Cyclin E was shown to require the sequential functioning of both Fbw7-alpha and Fbw7-gamma. The alpha isoform promoted the prolyl cis/trans isomerization of Cyclin E, via Pin1, priming the substrate for binding and ubiquitination by SCF[Fbw7-gamma] (69). Recently, utilizing specific Fbw7 isoform-null mutations, Grim *et al.* published that Fbw7-alpha may be the major isoform that mediates the stability of Cyclin E, c-Myc, and SREBP1 (70). Unfortunately, substrate recognition by Fbw7 may be more complex than simple
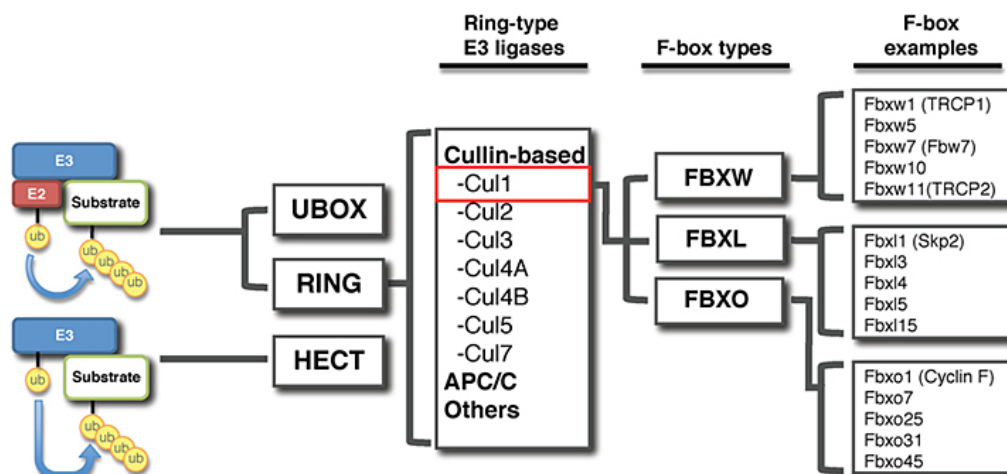
Figure 3. Illustration of the various types of E3 ligases and F-box proteins. E3 ubiquitin ligases are categorized into three major classes: U-box-type, Ring-finger-type, and HECT-type. U-box- and Ring-finger-type ligases function by bridging the interaction between the E2 enzyme and the substrate. HECT-type ligases are capable of directly conjugating ubiquitin onto the target protein. Ring-finger E3s are further divided into subfamilies including those that are cullin-based as well as the anaphase-promoting complex/cyclosome (APC/C). The SCF complex is a Cul1-based E3 ligase that consists of four components, Skp1, Cul1, Rbx1, and a variable subunit F-box protein. The F-box protein serves as a substrate recognition subunit for the SCF ligase by interacting with target proteins via its c-terminal binding domains. These F-box proteins fall into three major classes based on the substrate interaction domain that is present. Categories include F-box proteins that contain WD40 repeats (FBXW), leucine rich repeats (FBXL), or other protein interaction domains (FBXO).

differences in isoform specificity. For example, present within all isoforms of Fbw7 is a D-domain that mediates dimerization of the F-box protein (71, 72). While the importance of Fbw7 isoform dimerization is still not entirely clear, it may also play a role in substrate specificity (71).

7. FBW7, A *BONA FIDE* TUMOR SUPPRESSOR

Characterized substrates recognized by Fbw7 include Cyclin E, c-Myc, c-Jun, and Notch-1, all well-known oncogenes involved in a variety of human tumors (3, 48, 73). Thus, Fbw7 is a recognized tumor suppressor whose mutation occurs in multiple neoplasms including breast cancer, colon cancer, and leukemia (20, 74-77). Approximately 6% of all primary human tumors harbor mutations in the *FBW7* gene with the greatest mutation rates found in cholangio-carcinoma and T-cell acute lymphoblastic leukemia (T-ALL) (approximately 30%) (48, 49). A high percentage of Fbw7 mutations are amino acid substitutions found within the WD40 domains of the protein. The three conserved arginine residues that make direct contact with phosphorylated substrates are "hotspots" that account for almost 43% of all tumor-derived mutations in the *FBW7* gene (78). Therefore, disruption of substrate binding plays a major role in the mutational loss of SCF[Fbw7] function. Interestingly, mutations in the isoform-specific N-terminus of Fbw7 are quite rare (approximately 6%) (78). This low rate of mutations would suggest that all three isoforms might play some role in the anti-cancer functions of Fbw7.

Fbw7 substrates are well documented in their roles in cancer development, however, the mechanisms by which Fbw7 suppress cancer formation is unclear. While the inhibition of c-Myc and Notch-1 by Fbw7 has been reported, the contributions of these regulations to the tumor suppressive function of Fbw7 still require substantial characterization (79-81). Interestingly, most, but not all, Fbw7-mediated primary tumors possess elevated levels of total or phosphorylated Cyclin E (66, 82). This observation has garnered a focused attention on Cyclin E as a possible key mediator of Fbw7-induced cancer initiation through its ability to advance genetic instability. Lengauer and colleagues demonstrated that loss of Fbw7 function promoted genome instability that could be suppressed by the additional depletion of Cyclin E (82). Furthermore, Cyclin E (T380A), a mutant that cannot be recognized by Fbw7, could induce genome instability more effectively than wild type Cyclin E (83). Moreover, mice with the murine equivalent of the T380A mutation exhibited chromosomal instability and increased spontaneous cancer development (84). Although evidence suggests that Cyclin E activity may play a central role in Fbw7-mediated tumorigenic initiation, a considerable amount of work is still needed to elucidate its exact role in the process. While dysregulation of this cyclin may be a major driving force, it is unlikely to be the sole driving force. Another plausible explanation is that different substrates of Fbw7 may promote the various stages of tumorigenesis. Therefore, accumulation of multiple substrates may play equally important or synergistic roles in the overall transformation phenotype associated with the loss of Fbw7 function. Another possibility may be that individual substrates may only have defined roles in specific types of cancers. While both these hypotheses require additional work to substantiate their validity, research has begun on the characterization of molecular routes by which Fbw7
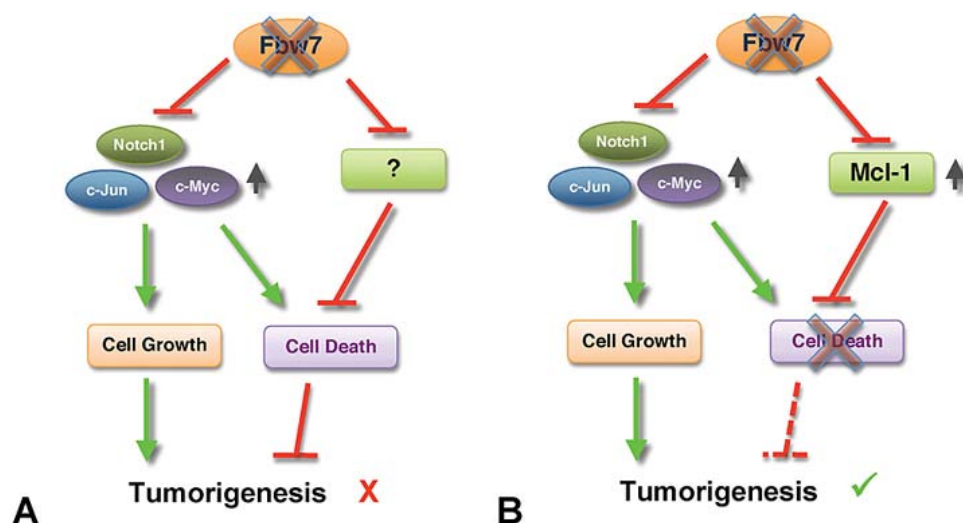
Figure 4. Fbw7 regulates cellular apoptosis by targeting the pro-survival factor Mcl-1 for degradation. (A) Loss of Fbw7 function leads to elevated levels of its many substrates including c-Myc, c-Jun, and Notch-1. While over-expression of these oncoproteins do give the cell a growth advantage, they also promote apoptosis, which in theory would inhibit tumorigenesis. Therefore, it was unclear how cells deficient in Fbw7 function can thrive in a setting of upregulated c-Jun, c-Myc or Notch-1. (B) We recently identified the pro-survival factor Mcl-1 as a novel target of SCF^{Fbw7}. Upon loss of Fbw7 function, cellular Mcl-1 levels are elevated. Higher expression of Mcl-1 in turn suppresses apoptosis caused by enhanced expression of c-Myc, c-Jun or Notch-1. This could then allow for uncontrolled cell growth and tumorigenesis.

activity may inhibit tumorigenesis in a known form of leukemia.

8. THE ROLE OF FBW7 IN SUPPRESSING T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

T-cell acute lymphoblastic leukemia (T-ALL) is a neoplastic disease of lymphoblasts committed to the T-cell lineage. While this type of cancer only accounts for a subset of acute lymphoblastic leukemias, it is often characterized as an aggressive disease that inevitably leads to poor clinical outcomes (85). One of the predominant obstacles that clinicians commonly face when treating T-ALL is that the patient set often becomes refractory to pharmacological intervention. Therefore, understanding the molecular mechanisms that promote T-ALL development becomes crucial as it would allow for more targeted therapies to be developed.

Mutations in the *FBW7* gene are commonly found in T-ALL (49). While *FBW7*-deficient mice are embryonic lethal at day 10.5 due to major developmental defects (38), conditional depletion of Fbw7 in mouse T-cells results in lymphomatogenesis (80, 81). In light of this, Fbw7 has been implicated as a novel tumor suppressor for T-ALL. Unfortunately, the molecular mechanism by which loss of Fbw7 function could promote T-ALL is unknown. It is also paradoxical how increased expression of known Fbw7 targets would advance this hematological malignancy. As stated previously, other known substrates of Fbw7 include c-Myc, c-Jun, and Notch-1, oncogenic proteins found over-expressed in a variety of human neoplasms including leukemia (48, 73). While elevated levels of these targets can accelerate cell growth (86),

which would promote tumorigenesis, over-expression of c-Jun, c-Myc, or Notch-1 can also result in cell death via up-regulation of the pro-apoptotic protein Bim-1 (87). Therefore, it is contradictory how cells lacking proper Fbw7 function can survive and prosper in a cellular environment where pro-apoptotic signaling may be prevalent (Figure 4A). However, recent work in our laboratory, as well as the Wertz group at Genentech, has begun to answer this question.

Mcl-1 is a member of the Bcl-2 family of proteins and suppresses the activities of the pro-apoptotic molecules Bim, Bax, and Bak (88). It is commonly found over-expressed in various malignancies and is extremely unstable (89). While its stability was initially demonstrated to require GSK3 activity (89), only recently did our lab, as well as Wertz *et al.*, establish that SCF^{Fbw7} targets Mcl-1 for ubiquitination and degradation in a GSK3-dependent manner (32, 33). Our studies further suggested a possible molecular mechanism by which elevated levels of Mcl-1 would provide protection from apoptosis induced by elevated c-Myc, c-Jun or Notch-1. Enhanced expression of Mcl-1 would then allow cells lacking Fbw7 function to survive in a cellular environment where pro-apoptotic signaling was high, such as in T-ALL (Figure 4B). Both studies also suggested a more clinical relevance to elevated levels of Mcl-1, whereas over-expression of the protein might determine the sensitivity of a tumor to known chemotherapeutics. Inuzuka *et al.* demonstrated that cells lacking Fbw7, which consequently had enhanced levels of Mcl-1, were resistant to the BCL2 antagonist ABT-737. Furthermore, reconstitution of Fbw7 function, or depletion of enhanced Mcl-1 levels, was able to restore the sensitivity of the cells to the pharmacological agent (33). This result

implied that elevated Mcl-1 levels allowed cells lacking Fbw7 to evade apoptosis. Wertz *et al.* further observed that loss of Fbw7 function, or increased Mcl-1 expression, in cancer cell lines promoted resistance to the anti-tubulin chemotherapeutics Taxol and Vincristine (32). These two studies have laid the groundwork for targeting Mcl-1 as a personalized treatment in cancers where Fbw7 function is lost.

9. THE SCF^BETA-TRCP E3 UBIQUITIN LIGASE

Another well-studied F-box protein is beta-TRCP (beta-transducin repeat containing protein). This FBP is highly conserved throughout evolution and has been characterized in multiple species including *Drosophila melanogaster* (Slimb), *Xenopus* (beta-TRCP), and mammals (beta-TRCP1 and beta-TRCP2). Structurally, similar to Fbw7, beta-TRCP contains an F-box motif at its N-terminus and seven substrate-binding WD-40 repeats at its C-terminus (90). Furthermore, this FBP recognizes a consensus DSGXXS degron in most of its target substrates (91). Similar to other SCF family ligases, the serine residues within this motif must first be phosphorylated before the substrate can be recognized by the E3 ligase. For example, the degron sequence in beta-catenin is phosphorylated by GSK3 and CKI before SCF^beta-TRCP can efficiently bind and mediate the ubiquitination of the target (92, 93). This F-box protein also exists as two paralogs in mammals, beta-TRCP1 and beta-TRCP2. Currently, the difference between these two proteins is not completely understood. Present studies indicate that these two paralogs have similar functions and may be redundant in their biological roles, helping to explain why beta-TRCP1 -/- mice develop normally with only minor defects in spermatogenesis, as beta-TRCP2 is still present and may compensate for the loss (94). Additionally, in beta-TRCP1 -/- MEFs, beta-TRCP1 was shown to contribute to substrate degradation, but its function was not completely required (94). To further complicate matters, both beta-TRCP1 and beta-TRCP2 also contain D-domains allowing for both homo-dimerization as well as hetero-dimerization. While this dimerization has been implicated in substrate specificity (95), further characterization of these interactions is still required.

It has been well demonstrated that beta-TRCP recognizes a wide range of cellular targets. Consequently, this FBP plays a vital role in regulating the activities of multiple biological processes. Some of the known beta-TRCP substrates including Wee1 (96), Cdc25A (97), and Emi-1 (98, 99) are cell cycle regulators. Consistent with this role, beta-TRCP1-/- mouse fibroblasts display decreased genetic stability and mitotic progression, primarily due to Wee1 and Emi-1 accumulation (96, 98, 99). Furthermore, beta-TRCP function has also been implicated in cell adhesion and migration through its phosphorylation-dependent regulation of the E-cadherin suppressor Snail (100). Moreover, recent research has also suggested that SCF^beta-TRCP may regulate glycolysis during the cell cycle (101).

10. BETA-TRCP, AN ONCOGENE OR A TUMOR SUPPRESSOR?

Multiple lines of evidence have already implicated the role of beta-TRCP in the promotion of various carcinomas when over-expressed. Cancers that display high levels of beta-TRCP include colon cancer (102), hepatoblastoma (103), pancreatic cancer (104), and melanoma (105). While a majority of these studies were performed using various cancer cell lines, oncogenic function of beta-TRCP has also been examined in multiple *in vivo* mouse models. Kudo *et al.* observed that tissue specific over-expression of exogenous beta-TRCP1 in mouse mammary epithelia lead to a significant increase in cell proliferation. Of the mice studied, 38% developed various carcinomas including mammary, ovarian, and uterine tumors (106). Consistent with these results, another group similarly reported that transgenic mice expressing wild type beta-TRCP1, or a dominant negative mutant of the protein (deltaF-beta-TRCP1), developed tumors as well (107).

However, the role of beta-TRCP as an oncogene is still debatable. In fact, based on certain known beta-TRCP substrates, it can be contradictory. In spite of this, various published reports do suggest that beta-TRCP functions primarily as an oncogene. First of all, this protein is mainly over-expressed in cancers. Secondly, genetic depletion of beta-TRCP1 shows no phenotype in mice (This, however, may be due to the existence of the second paralog with redundant function.) (94). Finally, multiple beta-TRCP substrates are known tumor suppressors. For example, IkappaB, a negative regulator of NFkappaB signaling, is a well-characterized target for phosphorylation-dependent beta-TRCP ubiquitination (108-113). NFkappaB is a transcription factor that targets multiple genes often associated with tumorigenesis and is commonly found to be over-activated in various inflammatory human cancers (114). Consistent with beta-TRCP regulating NFkappaB via IkappaB degradation, melanoma cell lines with enhanced beta-TRCP expression display elevated NFkappaB activity (105, 115). Recent work has also shown that the FOXO3 tumor suppressor is a target of beta-TRCP1 as well (116). In an orthotopic breast tumor mouse model, Tsai *et al.* observed that cells over-expressing beta-TRCP1 demonstrated increased tumorigenic activity. Furthermore, immunohistological analysis of the xenograft tumor samples confirmed an inverse correlation between beta-TRCP1 levels and FOXO3 expression (116). Beta-TRCP1 mediating the degradation of the tumor suppressor Repressor Element (RE1)-Silencing Transcription factor (REST) is yet another example of its function as an oncogene. In human mammary epithelial cells, re-introduction of a non-degradable mutant of REST could rescue the transformation caused by the over-expression of exogenous beta-TRCP (117).

While uncommon, mutations in beta-TRCP have also been observed in various cancers, which would imply a tumor suppressive role for this F-box protein. For example, a point mutation (F462S) in the WD40 substrate-

binding domain of beta-TRCP was identified in a gastric cancer cell line (118). In addition, mutations in this F-box protein have also been observed in prostate cancer (119) and in breast cancer (120). However, loss of function of beta-TRCP is rare in human carcinomas. Therefore, beta-TRCP most likely plays a more prominent role as an oncogene, with select situations where it may function as a tumor suppressor. One example of beta-TRCP possibly functioning as a tumor suppressor can be found in its regulation of the oncogene beta-catenin. Although up-regulation of beta-catenin is seen in many types of cancers, its enhanced half-life is often associated with mutations in the kinases that phosphorylate it (APC and axin) rather than in its E3 ligase (37, 92, 112, 121-124). Other beta-TRCP substrates that would also provide an oncogenic gain-of-function when over-expressed would include Cdc25 and Emi-1. While both of these proteins are elevated in different human cancers (125, 126), the link between defective beta-TRCP and their elevated expression is unknown. Accordingly, while largely oncogenic in nature, beta-TRCP may also function as a tumor suppressor under certain cellular contexts.

As with all E3 ligases, further identification of novel substrates will give researchers a more genuine understanding of the complex role these ligases may have in carcinogenesis. In the case of beta-TRCP, discovery of additional putative substrates would shed light on its function as either an oncogene or a tumor suppressor. Recent work in our laboratory has begun to answer this puzzling question with the discovery of Mdm2 as a novel substrate for the E3 ligase activity of beta-TRCP (43).

11. THE MDM2 ONCOPROTEIN, A NEGATIVE REGULATOR OF THE P53 TUMOR SUPPRESSOR, IS A NOVEL SUBSTRATE OF SCF[BETA-TRCP]

Dysregulation of the p53 tumor suppressor is found in a majority of all human tumors (127). This protein serves as a transcription factor that activates multiple downstream genes that promote cell cycle arrest (128-131) and apoptosis (132) upon genotoxic stress (133). Its function has been considered so critical for maintaining genomic integrity upon DNA damage that it has often been referred to as the guardian of the genome. Understanding its mechanisms of regulation is therefore important when considering anti-cancer regimens in patient sets where p53 function is compromised.

Mdm2 is one of the best-characterized negative regulators of the p53 pathway. It does so by promoting the ubiquitination and subsequent destruction of p53 (134). This is important as uncontrolled activation of the p53 pathway can result in premature senescence or apoptosis (135, 136). Interestingly, mice depleted of Mdm2 are embryonic lethal due to elevated levels of p53, suggesting a significant physiological role to this level of regulation (137). Prior studies have also demonstrated that Mdm2 undergoes rapid proteolysis upon DNA damage (138). Unfortunately, the mechanism of Mdm2 degradation is largely unknown. It was previously thought that auto-ubiquitination was the primary means by which Mdm2

proteolysis was regulated upon DNA damage (138). This, however, may not be the case. Recent studies have demonstrated that the E3 ligase activity of Mdm2 is not required for its degradation (139). This finding suggests that an unidentified E3 ligase might promote Mdm2 destruction.

Our laboratory recently reported a novel mechanism where Mdm2 is ubiquitinated by SCF[beta-TRCP] leading to the degradation of Mdm2 by the 26S proteasome (Figure 5) (43). As F-box proteins only recognize their putative substrates when phosphorylated, CKI was also identified as the modifying enzyme. Our studies demonstrated that upon treatment with DNA damaging agents, CKI translocates into the nucleus where it phosphorylates Mdm2, allowing beta-TRCP to recognize the target substrate (Figure 5). Consistent with beta-TRCP targeting Mdm2 in a CKI-dependent manner, depletion of endogenous beta-TRCP, or pharmacological inactivation of CKI, led to a marked increase in the steady state levels of Mdm2 (43). Moreover, our results also demonstrated that knockout of beta-TRCP resulted in changes in p53 expression oscillation upon DNA damage, suggesting that this regulation has physiological significance to the p53 DNA damage response pathway. Interestingly, while multiple CKI phosphorylation sites were identified on Mdm2 by mass spectrometry, mutation of any single site could not impair CKI phosphorylation and beta-TRCP binding. Instead, mutations of multiple sub-optimized degron sequences were necessary to sufficiently impair the proteolysis process. This mechanism is similar to that seen for the beta-TRCP substrate Gli3 (45) and the Fbw7 substrate Sic1. For Sic1, nine sub-optimized degron sequences are present on the protein with the phosphorylation of six being required to initiate proteolysis (44). Our findings suggest that similar to Sic1, Mdm2 requires a certain threshold phosphorylation before it can be adequately recognized by beta-TRCP. With increased phosphorylation above this threshold, Mdm2 becomes less stable due to increased binding to the E3 ligase. Physiologically, our studies also insinuate that dysregulation of Mdm2 degradation could potentially up-regulate the protein to disease state levels commonly seen in many types of carcinomas (140). With loss of beta-TRCP function, Mdm2 would become stabilized and subsequently promote p53 destruction. Loss of p53 function would in turn facilitate tumor progression. In summary, the findings by Inuzuka *et al.* provide insight into a molecular mechanism that would explain the frequent over-expression of Mdm2 observed in various types of human cancers. Furthermore, it also provides the rationale for the development of new anti-cancer therapeutics that target beta-TRCP or CKI in patients displaying elevated levels of Mdm2.

12. CONCLUSIONS AND PERSPECTIVES

With further identification of putative physiological substrates for both Fbw7 and beta-TRCP, clinicians will continue to gain deeper insight into cancers that develop due to mutations or over-expression of these F-box proteins. With a greater understanding of their
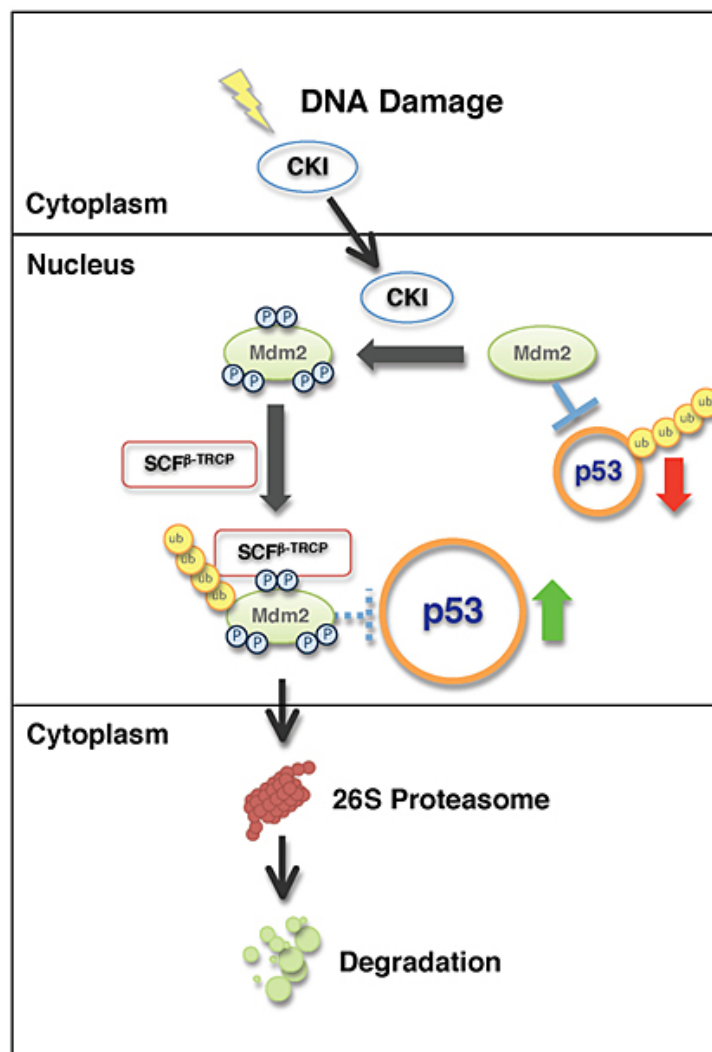
Figure 5. Multi-site phosphorylation of Mdm2 by CKI in response to DNA damage promotes Mdm2 ubiquitination and destruction by SCF[beta-TRCP]. In unstressed cells, p53 levels are maintained due to its interaction with Mdm2, which promotes the ubiquitination and proteolysis of p53. Upon DNA damage, CKI translocates to the nucleus where it multi-site phosphorylates Mdm2. This modified Mdm2 species is then recognized and bound by SCF[beta-TRCP]. The E3-ligase then in turn poly-ubiquitinates Mdm2 and promotes its degradation by the 26 proteasome.

substrate networks, scientists will have many more rationalized targets for pharmacological intervention. While the study of Fbw7 and beta-TRCP is still expanding, our current knowledge of these E3 ligases has already provided us with appealing therapeutic targets. With the identification of the anti-apoptotic protein Mcl-1 as a novel substrate of Fbw7 (32, 33), we now have a knowledgeable foundation and rationale to target this protein when treating Fbw7-deficient cancers. With the recent discovery that beta-TRCP can promote Mdm2 degradation in a CKI-dependent manner (43), we can now consider the possibility of targeting beta-TRCP or CKI in cancers where p53 function may be compromised.

While targeting the UPS in cancer therapy is still in its infancy, there have been great strides in defining

therapeutic targets within the system. Currently, the proteasome inhibitor bortezomib (Velcade, Millenium Pharmaceuticals) has been approved by the FDA and effectively used in the treatment of specific types of myelomas (141). Due to its success, second-generation proteasome inhibitors are also currently under development, including carfilzomib (Onyx Pharmaceuticals), an irreversible proteasome inhibitor that has shown promising results (142). Furthermore, an inhibitor of the cullin neddylation process MLN4924 has been developed as a more specific intervention method for inhibiting cullin-based ligases (including SCF-type E3 ligases) (143). In contrast to more general proteasome inhibition methods, targeting of specific E3 ligases may eventually prove to be more efficacious while also avoiding unwanted side effects. Blockage of broad proteolysis would

lead to the up-regulation of all cellular proteins destined for destruction, while E3 specific antagonizers would only stabilize a subset of proteins. With the development of Nutlins, small molecules designed to hinder the binding between p53 and Mdm2 (144, 145), it seems that E3 ligase specific blockage may well be on its way to becoming a viable treatment option in battling cancer and other diseases. While Nutlins impede ligase/substrate interaction, other compounds have also been discovered that inhibit E3 ligase activity. One family of compounds, HLI98, was recently reported to prevent Mdm2-mediated ubiquitination of p53 both *in vitro* and *in vivo* (146). Bioengineering has also been utilized to create E3 ligases with increased activity towards their substrates (147). With the discovery of these molecules, it is now encouraging to develop inhibitors for any given F-box protein with oncogenic activity.

Unfortunately, there are still many obstacles to overcome before targeting E3 ligases becomes a viable treatment option. One such obstacle is the question of selectivity. Any new therapeutic must only target the pathological cells but not the healthy ones. Furthermore, manipulating a specific E3 ligase may still have consequences beyond our expectations. While targeting one E3 ligase may prove to be more efficacious than general proteasome inhibition, multiple proteins, in addition to the primary therapeutic target, would still be affected as most E3 ligases regulate the stability of numerous targets. Nevertheless, continued research on the UPS will undoubtedly lead to the discovery of new 'druggable' targets that may promote the development of more specific, less toxic and more efficacious anti-cancer therapeutics.

13. ACKNOWLEDGEMENTS

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