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Review

Novel roles of Skp2 E3 ligase in cellular senescence, cancer progression, and metastasis

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Abstract

S-phase kinase-associated protein 2 (Skp2) belongs to the F-box protein family. It is a component of the SCF E3 ubiquitin ligase complex. Skp2 has been shown to regulate cellular proliferation by targeting several cell cycle-regulated proteins for ubiquitination and degradation, including cyclin-dependent kinase inhibitor p27. Skp2 has also been demonstrated to display an oncogenic function since its overexpression has been observed in many human cancers. This review discusses the recent discoveries on the novel roles of Skp2 in regulating cellular senescence, cancer progression, and metastasis, as well as the therapeutic potential of targeting Skp2 for human cancer treatment.

Key words Skp2, p53, RhoA, cellular senescence, metastasis, cancer therapy

Ubiquitin is a highly evolutionarily conserved protein consisting of 76 amino acids. In eukaryotic cells, the ubiquitin proteasome system (UPS) mediates the majority of protein degradation, which plays important roles in the regulation of multiple responses to diverse signals during development and metabolism[1]. Posttranslational modification of proteins with ubiquitin is mediated by three enzymes: the E1 activating enzyme, the E2 conjugating enzyme, and the E3 ligase (Figure 1). Ubiquitination is a covalent reaction that attaches ubiquitin(s) to one or more lysine residues in a protein. In the human genome, 2 E1s, roughly 50 E2s, and 600 E3s have been identified[2]. The substrate specificity in the UPS is determined by the E3 ligase, which is subjected to regulation at multiple levels. Deregulation of E3 ligase has been implicated in human diseases such as cancer.

S-phase kinase-associated protein 2 (Skp2) is an F-box protein, which constitutes one of the four subunits of the Skp1-Cullin-1 (Cul-1)-F-Box (SCF) ubiquitin E3 ligase complex. Earlier studies have shown that Skp2 regulates cell cycle progression and proliferation by targeting ubiquitination and degradation of its substrates, such as cell cycle inhibitor p27[3,4]. Subsequent studies with human cancer samples revealed that Skp2 is overexpressed in a variety of human cancers and is inversely correlated with p27 levels, suggesting that Skp2 overexpression may have essential functions in human cancer development[5,6]. Other studies using xenograft mouse tumor models have supported the oncogenic role of Skp2 in cancer development[7-9]. Moreover, recent work using a *Skp2*-deficient mouse model has revealed that Skp2 is required for cancer development in multiple tumor-promoting conditions, including *PTEN*, *ARF*, and *pRB* inactivation[10,11].

In this review, we will summarize recent findings on the novel roles of E3 ligase Skp2 in human cancers, with a particular emphasis on cellular senescence, cancer progression, and metastasis.

Skp2 Is a Member of the F-box Protein Family

The function of the SCF E3 ligase is to facilitate the transfer of ubiquitin from E2 ubiquitin conjugating enzymes to protein substrates. Currently, 68 F-box proteins have been identified in the human genome. Although structurally diverse, they all contain an F-box motif consisting of approximately 45–50 amino acids[12].

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In addition to the F-box motif, all F-box proteins have a protein-protein interaction domain for substrate binding and recognition that falls into the following three classes (Figure 1) [13,14]: (1) WD40 repeat-containing F-box proteins (FBXWs), such as β-TRCP and FBXW7; (2) leucine-rich repeats (LRRs)–containing F-box proteins (FBXLs), including Skp2; and (3) F-box proteins containing other diverse domains (FBXOs), which include proteins with other domain types in the C-terminal region. Both genetic and biochemical analyses have demonstrated that the SCF E3 ligase targets a variety of important proteins for ubiquitin-dependent proteasome degradation. Interestingly, phosphorylation of the substrates on either serine or threonine is required for SCF-mediated protein degradation, which is mediated through the WD40 and LRR repeats.

Among the large number of F-box proteins identified in the human genome, only a few, such as Skp2, β-TRCP, and FBXW7, have been extensively studied, including the identification of their substrates.

Skp2 Targets Proteins for Ubiquitination and Degradation

As mentioned above, Skp2 is a critical component of $Skp2^{SCF}$ ubiquitin E3 ligase, one of the best-characterized SCF complexes. Skp2 was first identified because of its overexpression in many cancer cell lines and its association with Skp1 and cyclin A/Cdk2/Cks1 [15]. A variety of Skp2 substrates, which are involved in multiple cellular processes such as cell cycle and apoptosis, have been identified [13]. The best-known Skp2 substrate is cell cycle inhibitor p27, which is targeted by $Skp2^{SCF}$ for ubiquitination and degradation when threonine 187, located at the C-terminal end of p27, is phosphorylated [16-18]. The binding and recognition of p27 as a substrate by Skp2 requires an accessory protein, Cks1, as *Cks1* deficiency prevents Skp2 from binding to p27, in turn leading to p27 up-regulation [19-21]. Strikingly, genetically engineered *Skp2* knockout mice and *Cks1*-deficient mice share similar phenotypes. Mouse embryonic fibroblasts (MEFs) from $Cks1^{-/-}$ and $Skp2^{-/-}$ mice both display reduced cell proliferation, accompanied by enhanced p27 protein expression [3]. Thus, it appears that the function of Cks1 is to facilitate Skp2-mediated substrate polyubiquitination. Both *in vitro* and *in vivo* evidence suggests that p27 is a critical and relevant Skp2 substrate for Skp2 functions. Double deficiency of *p27* and *Skp2* rescues the cell proliferation defect observed in $Skp2^{-/-}$ MEFs as well as the reduced organ size and body weight observed in *Skp2*-deficient mice [4]. Importantly, there is an inverse correlation between Skp2 and p27 expression in human cancers. Notably, Skp2 is found to be overexpressed in various human cancer samples and associated with poor prognosis

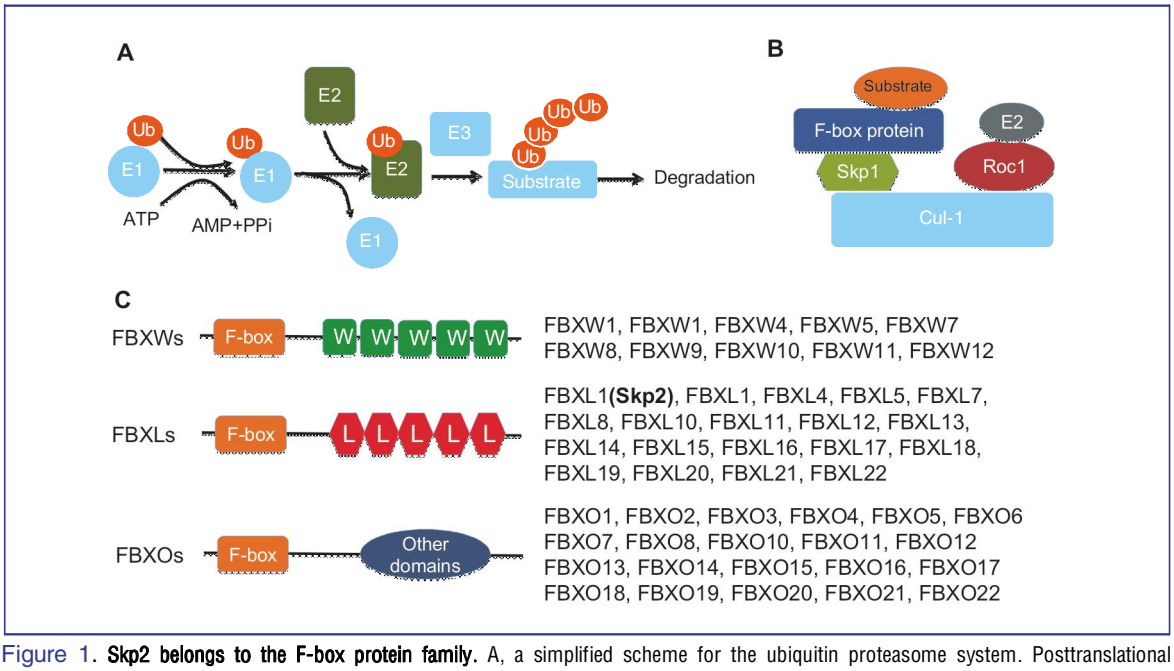

Figure 1. **Skp2 belongs to the F-box protein family.** A, a simplified scheme for the ubiquitin proteasome system. Posttranslational modification of a protein with ubiquitin is mediated by three enzymes: the E1-activating enzyme, the E2-conjugating enzyme, and the E3 ligase. B, SCF E3 ligase complex is composed of Cul-1, Skp1, Roc1, and an F-box protein, such as Skp2. C, F-box proteins can be divided into three classes, WD40 repeat-containing F-box proteins (FBXWs), leucine-rich repeats (LRRs)–containing F-box proteins (FBXLs) including Skp2, and F-box proteins containing other diverse domains (FBXOs).

and is accompanied by p27 down-regulation[5,6,22-25].

Although p27 is a critical target of Skp2, many additional substrates of $Skp2^{SCF}$ have been identified, and the relevance of these proteins to Skp2 function remains to be determined. Many of these proteins, such as p21 [26,27], p57[28], E2F-1[29], MEF[30], p130[31,32], Tob1[33], cyclin D [27], cyclin E [34], Smad4 [35], Myc [36,37], B-Myb [38], and RASSF1A[39], are cell cycle regulators. In addition, Skp2 also targets apoptosis regulators such as Myc [36,37] and Foxo[36,37] for protein degradation. Moreover, Skp2 targets many other proteins with diverse functions for degradation, including Orc1p [40] and Cdt1 [41,42], Rag-2 [43], BRCA2 [44], Cdk9[45], MKP1[46], and UBP43[47]. These results suggest that Skp2 plays a role not only in cell cycle progression and apoptosis, but also in a wide range of other biological processes. To understand the relevance of these substrates to Skp2 function, genetic mouse models will be required for further study.

Overexpression of Skp2 is frequently observed in human cancers, and accumulating evidence suggests that Skp2 plays a proto-oncogenic role both *in vitro* and *in vivo*. Skp2 is able to cooperate with H-RasG12V to induce cellular transformation in soft agar assays and tumor formation assays in nude mice[48,49]. Although overexpression of Skp2 in the T-cell compartment is not sufficient to induce T-cell lymphomas, the combined overexpression of Skp2 with N-ras leads to T-cell lymphomas with shorter latency and higher penetrance and results in a significant decrease in mouse survival compared to the *N-ras* transgenic mouse alone[49], suggesting a role for Skp2 in the progression of T-cell lymphoma. Moreover, overexpression of Skp2 in mouse prostate leads to prostate intraepithelial neoplasia (PIN)[50]. In line with these observations, our recent study showed that overexpression of Skp2 in prostate cancer cells significantly promotes prostate cancer cell growth and tumorigenesis in a xenograft tumor model[7], whereas overexpression of the Skp2 S72A mutant, which is unable to be phosphorylated by Akt, could not induce these same effects[7]. Therefore, our study underscores the critical role of Akt-mediated Skp2 phosphorylation at Ser 72 in the regulation of Skp2SCF activity and oncogenic transformation.

Skp2 Suppresses Cellular Senescence Induced by Oncogenic Stimuli Independent of ARF/p53

Cellular senescence, first described by Hayflick *et al*. in 1961[51,52], is an irreversible form of cell cycle arrest that can be triggered by a variety of insults via two mechanisms: genetic reprogramming and in response to damaging conditions, which will lead to replicative senescence and premature senescence, respectively[53-55]. In addition to cultivation-induced proliferative exhaustion, several other stress stimuli, including telomere dysfunction, DNA damage accumulation, and genotoxic stress, have been shown to induce replicative senescence. Premature cellular senescence can be triggered by the overexpression of several oncogenes (also termed oncogene-induced senescence, OIS), such as $HRas^{G12V}$ and $Braf^{E600E}$ [55-57], as well as loss of tumor suppressor PTEN (also termed PTEN-loss-induced senescence, PICS) [55,58,59] or other tumor suppressors, such as neurofibromatosis type 1 (NF1) and Von Hippel-Lindau (VHL) [59-61]. Recent studies suggest that cellular senescence can serve as an important tumor-suppressive barrier to restrict tumor development *in vivo*[55,59,62-67].

Tumor suppressor p53 has been shown to be essential in inducing cell cycle arrest, apoptosis, and senescence in response to various stress signals[68-72]. Induction of p53 in response to these stress stimuli leads to cellular senescence both *in vitro* and *in vivo*[56,63,73]. On the contrary, silencing or inactivation of *p53* by viral oncoproteins, such as SV40 large T antigen or HPV-16 E6 protein, suppresses senescence response[74-77]. Importantly, genetic inactivation of *p53* in mice eliminates the senescence resulting from overexpression of oncogenic $HRas^{G12V}$ or $BRaf^{V600E}$, which ultimately leads to promotion of cancer progression in mouse models[56,65,78-80]. Thus, these findings indicate an important role of p53 in the regulation of cellular senescence.

Although cellular senescence critically depends on the ARF/p53 pathway, emerging evidence suggests that cellular senescence can also be triggered in an ARF/p53-independent manner. For example, acute inactivation of *VHL*, a tumor suppressor that is frequently mutated in many human cancers[81], triggers cellular senescence *in vitro* and *in vivo*. Surprisingly, the cellular senescence resulting from loss of VHL is dependent on pRB activation and p400 reduction[61]. Interestingly, *Skp2* mRNA levels are significantly reduced when *VHL* is inactivated, thus leading to increased p27 expression[61]. This implies that Skp2 might also play a direct role in cellular senescence. In line with this notion, Skp2 down-regulation through the overexpression of the human T lymphotropic virus type 1 (HTLV-1) Tax protein also results in cellular senescence[82].

Our recent work provides strong evidence that Skp2 indeed plays a direct role in cellular senescence. Our study reveals that genetic *Skp2* inactivation alone does not induce cellular senescence response. However, the combined inactivation of *Skp2* and tumor suppressors *PTEN* or *ARF* results in cellular senescence both *in vitro* and *in vivo*[10]. Consistent with previous observations showing that Skp2 mediates p21 and p27 degradation[3,4,26], p21 and p27 protein levels are increased in $Skp2^{-/-}$ MEFs[10]. Interestingly, we found that *Skp2* deficiency

sensitizes cancer-prone cells expressing Ras and E1A oncoproteins or partially lacking tumor suppressor *PTEN* to senescence[10]. Such a senescence response triggered by inactivation of *Skp2* in the presence of powerful oncogenic signals, even when the p19ARF/p53 response is evaded, suggests that Skp2 regulates a p53/ARF-independent senescence pathway. Furthermore, compound mutant mice deficient in both *PTEN* and *Skp2* (*PTEN*$^{+/-}$; *Skp2*$^{-/-}$) are strongly protected from cancer[10]. Adrenal tumor formation and lymphoadenophathy are profoundly inhibited in these compound mutant mice as compared to the *PTEN*$^{+/-}$ mice. Similar results are observed in mice deficient for *PTEN* in the prostate and those deficient for tumor suppressor *p19ARF*[10]. In these two mouse models, *Skp2* inactivation in combination with deficiency of *PTEN* or *p19ARF* renders mice resistant to cancer. Importantly, a significant increase in senescent cells and a reduction in proliferation rate are observed within the pre-tumoral tissues from the cancer-resistant organs, particularly the lymph nodes and prostate, as compared to those of mice expressing wild-type Skp2. Interestingly, the senescence response triggered by *Skp2* inactivation in combination with *PTEN* inactivation or *ARF* loss does not result in *p53* activation or DNA damage response (Figure 2). Our data suggest that combined deficiency for *Skp2* and *PTEN* or *ARF* leads to induction of cell cycle inhibitors p27 and p21 and endoplasmic reticulum stress protein ATF4, which can synergistically contribute to senescence response [10]. Collectively, these findings suggest that *Skp2* regulates a novel p19ARF/p53- independent senescence response to promote tumorigenesis.

Skp2 Regulates Cell Migration and Metastasis by Affecting *RhoA* Transcription in Cooperation with Myc-Miz1-P300 and Independent of SCF-Skp2 E3 Ligase Activity

Metastasis is a complex process that accounts for a majority of death from cancer. Many key players involved in cell migration, invasion, and metastasis have been identified. RhoA, a member of the Rho family of GTPases, plays important roles in numerous biological

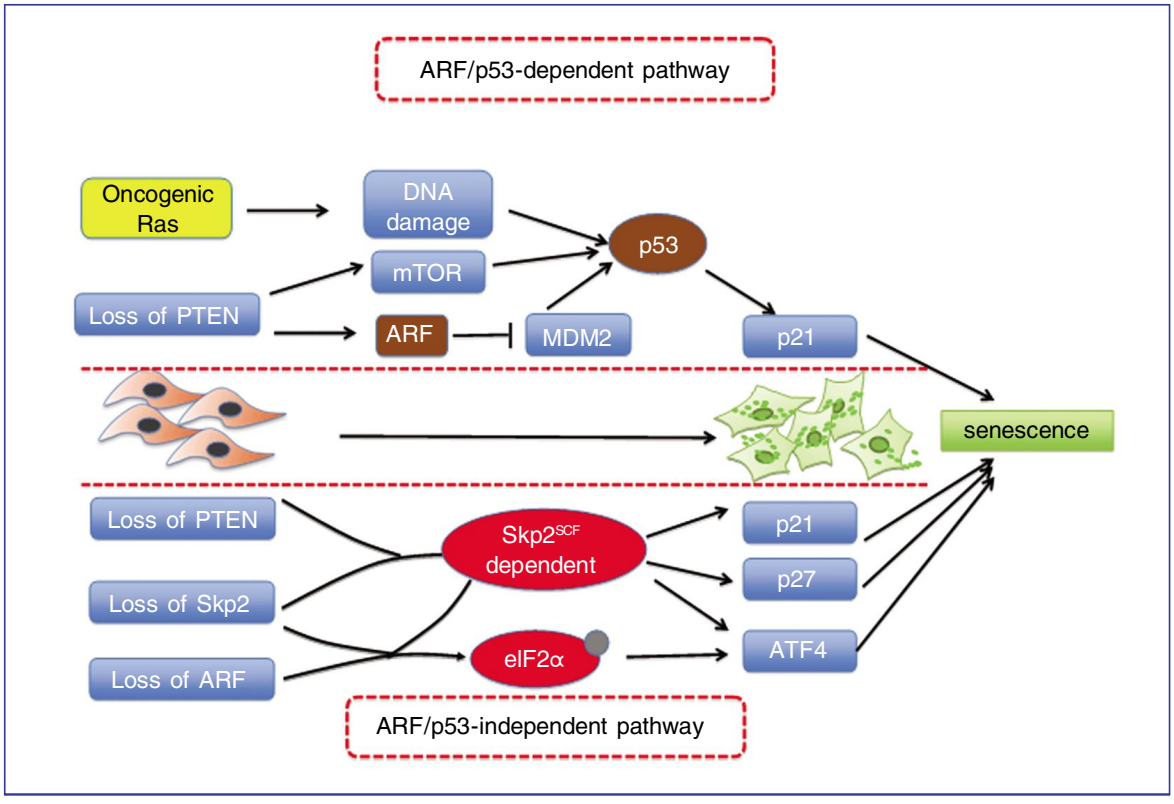

Figure 2. **ARF/p53-dependent and -independent pathways for cellular senescence.** Premature cellular senescence can be triggered by overexpression of several oncogenes, such as *HRas*^{G12V}, as well as loss of tumor suppressors, such as *PTEN*, *NF1*, *VHL*, and *ARF*. Overexpression of *Ras* or loss of *PTEN* leads to ARF/p53 activation and cellular senescence. However, inactivation of *Skp2* in combination with *PTEN* or *ARF* inactivation leads to an induction of *p21*, *p27*, and *ATF4* in an ARF/p53-independent manner.

processes such as migration and invasion and has been implicated in cancer metastasis[83]. Although no mutation of RhoA has been found in human cancers[84,85], RhoA mRNA as well as protein levels are up-regulated in various human cancers[86-88]. However, how *RhoA* transcription is regulated remains largely unknown. Our recent work identified Skp2 as a key player in cell migration and invasion and uncovered transcriptional machinery, Skp2-Miz1-cMyc-p300, that is important for the regulation of *RhoA* transcription.

Skp2 overexpression has been reported to be associated with cancer progression and metastasis[7,89-93]. Importantly, our recent work established a direct role for Skp2 in cell migration, invasion, and metastasis (Figure 3)[7,94]. We found that *Skp2*-deficient cells (*Skp2*$^{-/-}$ MEFs or *Skp2* knockdown cancer cells) were severely impaired in cell migration and invasion, whereas cells with Skp2 overexpression had significantly increased cell migration and invasion activities[7,94]. Moreover, we have previously shown that Akt-mediated phosphorylation of Skp2 at serine 72 and subsequent cytosolic relocalization of phosphorylated Skp2 could promote cell migration[7]. Specifically, restoration of a cytosolic Skp2 mutant, Skp2-NES, could rescue the migration defects observed in the *Skp2*$^{-/-}$ MEFs, suggesting that cytosolic Skp2 may play an important role in cell migration and invasion (Figure 3). Recently, we identified a transcriptional complex of Skp2, including Myc, Miz1, and p300, that is important for the regulation of *RhoA* transcription. *Skp2*$^{-/-}$ MEFs or *Skp2* knockdown cells showed a profound decrease in both RhoA mRNA and protein levels, whereas Skp2 overexpression induced RhoA protein expression, which further increased when Myc was co-expressed[94]. The restoration of RhoA expression partially rescued the migration and invasion defect in *Skp2*$^{-/-}$ MEFs and *Skp2* knockdown cells, indicating that RhoA is an important downstream effector for Skp2 in cell migration[94]. Using a chromatin immunoprecipitation (ChIP) assay, we demonstrated that Skp2 is recruited to the *RhoA* promoter by Myc, as *Myc* knockdown prevents Skp2 from binding to the *RhoA* promoter, whereas Skp2 mutants defective in Myc binding fail to activate *RhoA* transcription[94]. These results suggest that the function of Skp2 in transcriptional regulation is critically dependent on Myc. Furthermore, we found that Skp2 recruits Miz1 and p300 to the Myc transcriptional complex to induce *RhoA* expression, and the recruitment of p300 and Miz1 to the *RhoA* promoter is impaired in *Skp2* knockdown cells[94]. Importantly, using a well-established breast cancer lung metastasis model generated by tail vein injection of MDA-MB-231 breast cancer cells, we demonstrated that knockdown of *Myc*, *Miz1*, or *Skp2* profoundly reduced metastasis to the lung whereas overexpression of Myc or Skp2 promoted lung metastasis, suggesting that the Myc-Skp2-Miz1 complex has essential functions in cell invasion and metastasis. Importantly, RhoA, Myc, Miz1, and Skp2 are all significantly correlated with human prostate cancer metastasis. Moreover, RhoA expression is strongly correlated with Myc, Skp2, and Miz1 expression in prostate cancer. Furthermore, we found there is also a positive correlation between Myc, Skp2, and Miz1. Taken together, these results demonstrate that Myc-Skp1-Miz1 has important functions in *RhoA* transcription and underscore the clinical relevance of the Myc-Skp2-Miz1 complex and *RhoA* expression in cancer metastasis (Figure 3). However, it is unclear whether Akt-mediated phosphorylation of Skp2 also contributes to Skp2-mediated recruitment of Miz1 and p300 and subsequent Myc-Skp2-Miz1 transcriptional complex formation.

Interestingly, the ability of Skp2 to regulate *RhoA* transcription appears to be independent of its SCF-Skp2 E3 ligase activity. We found that both wild-type Skp2 and Skp2-LRR mutant, which is devoid of the amino terminus and F-box domain and defective in E3 ligase activity, similarly interacted with Myc, p300, and Miz1 and cooperated with them in the up-regulation of *RhoA* transcription[94]. In addition, the Skp2-LRR mutant was able to promote breast cancer cell metastasis to the lung as efficiently as wild-type Skp2 but was less efficient at promoting cell cycle progression, cell proliferation, and primary tumor formation compared to wild-type Skp2. Taken together, these data suggest that Skp2^{SCF} E3 ligase activity is required for cell cycle progression and tumorigenesis but is dispensable for *RhoA* gene expression and cancer metastasis.

Targeting Skp2 for Cancer Therapy

Emerging evidence suggests that targeting Skp2 in human cancers may offer therapeutic benefits in various cancers, as Skp2 has been shown to be overexpressed in multiple types of human cancers. Recently, three groups reported that Skp2 is required for tumorigenesis in mouse models in the context of BCR-ABL overexpression, *PTEN* loss, or *pRB* inactivation[44,62,95], suggesting that targeting Skp2 may be very effective for treatment of many types of human cancers. In recent years, mounting evidence suggests that cellular senescence serves as a crucial barrier in restricting tumor progression. Thus, it has been proposed that approaches to enhance the pro-senescence response may be effective therapeutic strategies for human cancer[55]. However, as *p53* is the most commonly mutated gene in human cancers, strategies targeting p53-dependent cellular senescence may not be applicable for tumors with *p53* loss or inactivation. Therefore, identification and targeting of *p53*-independent cellular senescence pathways may be the key for the success of

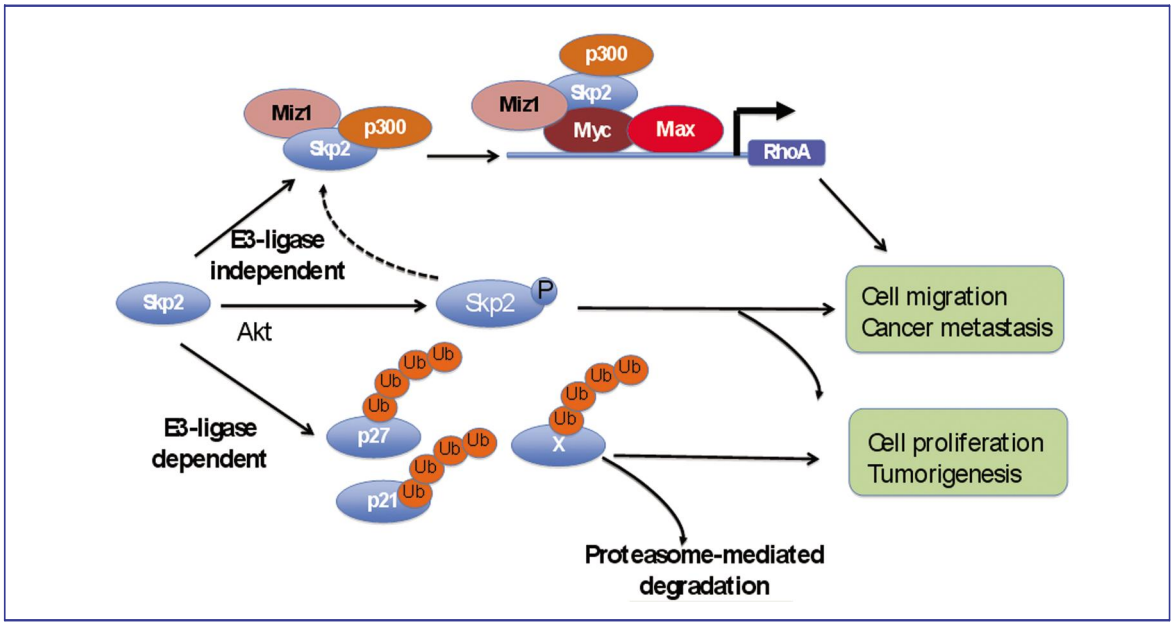

Figure 3. **E3-dependent and E3-independent functions of Skp2.** Skp2 E3 ligase can target various proteins, such as p27, for proteasome-mediated degradation. However, Skp2 can also function independently of its E3 ligase activity. Skp2 can be phosphorylated by Akt and promote cell migration and metastasis. In addition, Skp2 can recruit Miz1 and p300 and form a transcriptional complex of Myc-Skp2-Miz1-p300 to regulate RhoA expression. Such activities of Skp2 do not require its E3 ligase activity.

pro-senescence therapy. As *Skp2* deficiency triggers p53-independent cellular senescence, targeting Skp2 may be an ideal approach for the treatment of advanced human cancers with *p53* inactivation. Furthermore, targeting Skp2 may also be a potential strategy to treat metastatic cancers, as we demonstrated that overexpression of Skp2 promotes cell migration and metastasis in cooperation with Myc-Miz1 to regulate *RhoA* transcription, whereas inactivation of Skp2-Myc-Miz1 restricts cancer metastasis.

Given that Skp2 plays an important role in tumorigenesis, the development of specific Skp2 inhibitors will offer novel therapeutics for the treatment of human cancer. Recently, two small molecules targeting other components of the $Skp2^{SCF}$ complex were identified: a small molecule (compound A) targeting $Skp2^{SCF}$ E3 ligase activity towards p27 ubiquitination [95], and MLN4924, a small molecule inhibitor targeting Nedd8-activating enzyme, thereby affecting Cul-1 neddylation and $Skp2^{SCF}$ complex formation [96]. The first study demonstrated that administration of this inhibitor causes cell arrest, apoptosis, and autophagy in leukemia cells, whereas the second study demonstrated that MLN4924 reduced Cul-1 neddylation, accompanied by inducing p27 accumulation, cell arrest, apoptosis, and senescence [96,97]. Furthermore, MLN4924 has potent effects on suppressing tumor growth *in vivo* using xenograft tumor models [96,97]. Thus, these studies clearly demonstrated that developing specific inhibitors to target Skp2 could offer novel and effective therapeutics against human cancer. We are in urgent need of such inhibitors to combat human cancers.

Conclusions and Perspective

Overexpression of Skp2 has been observed in many types of human cancers. Recent studies demonstrate that Skp2 overexpression promotes cancer progression and metastasis, whereas loss of Skp2 function inhibits these processes. In addition, *Skp2* deficiency in tumor cells can trigger p53-independent senescence response. Collectively, these studies suggest that targeting Skp2 may be an ideal therapeutic strategy for human cancer, therefore establishing a rationale for designing small molecule inhibitors of Skp2 for the treatment of human cancers. An alternative approach to reduce Skp2 expression and activity as a means of cancer treatment is to target the transcription, protein stability, and SCF complex formation of Skp2. *Skp2* gene expression is regulated by the Notch, IKK/NF-κB, or Akt signaling pathways, thus inhibition of these oncogenic pathways is expected to shut down *Skp2* gene expression. Importantly, small molecules targeting these pathways are currently in clinical trials. In addition, the stability of

Skp2 protein is positively regulated by Akt and CDK2 activity. Thus, small molecule inhibitors targeting Akt and CDK2 will be able to trigger rapid Skp2 degradation, in turn inhibiting cancer development. Furthermore, the $Skp2^{SCF}$ E3 ligase can function to target protein degradation only when this complex is properly formed. Thus, targeting Cul-1 neddylation can efficiently disrupt the $Skp2^{SCF}$ complex formation and can lead to an impaired SCF E3 ligase activity. One approach to target $Skp2^{SCF}$ complex formation is to use MLN4924, a small molecule inhibitor known to target Cul-1 neddylation.

Although there is significant progress in the field of Skp2 research, future studies need to be conducted to address several important questions. First, although many substrates have been identified for $Skp2^{SCF}$, it remains to be determined which substrate is specifically required for Skp2-mediated cell proliferation, apoptosis, and tumorigenesis. Second, CAND1 has been shown to inhibit $Skp2^{SCF}$ complex by binding to unneddylated Cul-1[98], but the exact roles of CAND1 in $Skp2^{SCF}$ complex formation and cancer progression remain unclear. It is conceivable that CAND1 may play a tumor suppressive role in human cancers. Third, the mechanism by which Akt-mediated Skp2 phosphorylation regulates cell migration needs further investigation. Fourth, it is unclear whether Skp2 is globally involved in cancer development in various human tissues. Lastly, there is no evidence for the requirement of Skp2 in cancer maintenance in various cancers. Future studies using multiple mouse models and *in vitro* approaches are required to address these challenging questions, which will ultimately lead to a more comprehensive understanding of how the $Skp2^{SCF}$ complex is regulated and its roles in cancer progression and metastasis.

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