

## Role of E3 ubiquitin ligases in lung cancer

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ligase inhibitors and pave the way for novel treatment strategies for cancer patients.

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**Key words:** E3 ubiquitin ligases; Lung cancer; Ubiquitin-proteasome system; Proteasome inhibitors; Bortezomib; Apoptosis; Gene regulation; DNA repair

**Core tip:** E3 ubiquitin ligases catalyze ubiquitination of proteins for degradation by the 26S proteasome. They are important for many biological processes including cell cycle regulation, proliferation and apoptosis. They are often overexpressed and deregulated in lung cancer, which contributes to cancer development. These processes underline their potential as anti-cancer targets. There is only one E3 ubiquitin ligase inhibitor in clinical trial. A better understanding of how E3 ubiquitin ligases regulate biological processes and of their exact role in carcinogenesis, will help to develop specific E3 ubiquitin ligase inhibitors to improve treatment strategies for cancer patients.

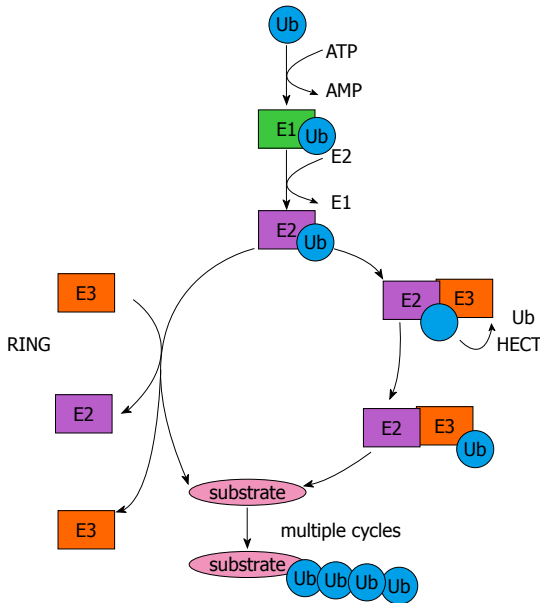
### Abstract

E3 ubiquitin ligases are a large family of proteins that catalyze the ubiquitination of many protein substrates for targeted degradation by the 26S proteasome. Therefore, E3 ubiquitin ligases play an essential role in a variety of biological processes including cell cycle regulation, proliferation and apoptosis. E3 ubiquitin ligases are often found overexpressed in human cancers, including lung cancer, and their deregulation has been shown to contribute to cancer development. However, the lack of specific inhibitors in clinical trials is a major issue in targeting E3 ubiquitin ligases with currently only one E3 ubiquitin ligase inhibitor being tested in the clinical setting. In this review, we focus on E3 ubiquitin ligases that have been found deregulated in lung cancer. Furthermore, we discuss the processes in which they are involved and evaluate them as potential anti-cancer targets. By better understanding the mechanisms by which E3 ubiquitin ligases regulate biological processes and their exact role in carcinogenesis, we can improve the development of specific E3 ubiquitin

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### INTRODUCTION

The ubiquitin-proteasome system (UPS) regulates multiple biological aspects of cell survival by mediating the degradation of targeted proteins and thereby maintaining cellular homeostasis<sup>[1]</sup>. In numerous cancer types, the deregulation of UPS components has been observed and their overexpression is often associated with chemoresistance and poor prognosis<sup>[2-5]</sup>. For example, the E3 ubiquitin ligase murine double minute 2 (MDM2), which is involved in the regulation of p53 levels, is frequently overexpressed in tumors and is predicted to be a nega-



**Figure 1** Ubiquitin molecules are covalently conjugated to targeted proteins in a three-step enzymatic cascade. First, an E1 ubiquitin-activating enzyme activates ubiquitin in an adenosine triphosphate (ATP)-dependent manner. Activated ubiquitin is then transferred to the E1 enzyme, followed by the transfer of ubiquitin to an E2 ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin protein ligase recognizes the target proteins and mediates the conjugation of one or more ubiquitin molecules to a lysine residue on the targeted proteins. Really interesting new gene (RING) E3 ubiquitin ligases mediate the direct transfer of ubiquitin from E2 to the targeted substrate whereas Homologous to the E6-associated protein (E6-AP) carboxyl terminus (HECT) E3 ubiquitin ligases first interact with the cognate E2, followed by linkage with ubiquitin and subsequent transfer of ubiquitin to the targeted substrate. AMP: Adenosine monophosphate.

tive prognostic marker for the development of several human cancers including breast carcinoma and prostate carcinoma<sup>[6-9]</sup>.

In the early 1980s, Ciechanover *et al.*<sup>[10]</sup> and Hershko *et al.*<sup>[11,12]</sup> obtained the initial understanding of ubiquitin-mediated protein degradation and identified several components of the ubiquitin system. A set of interconnected studies between 1984 and 1990 revealed the biological significance of protein degradation mediated by the ubiquitin system<sup>[13,14]</sup>. The mechanism by which ubiquitin molecules are covalently attached to targeted proteins can be delineated as a three-step enzymatic cascade (Figure 1). First, an ubiquitin-activating enzyme (E1) mediates the activation of the carboxyl-terminal glycine residue of ubiquitin in an ATP-dependent manner<sup>[10,15]</sup>. With the formation of a thiolester linkage, the activated ubiquitin is then transferred to E1 followed by the transfer of ubiquitin to a thiol site of an ubiquitin-conjugating enzyme (E2)<sup>[16]</sup>. Finally, an ubiquitin protein ligase (E3) confers substrate specificity by recognizing the target proteins and mediating the conjugation of (a) ubiquitin molecule(s) to a lysine residue on the targeted protein *via* an isopeptide bond<sup>[12]</sup>. Subsequently, the targeted protein is marked for degradation by the ATP-dependent 26S proteasome.

The addition of ubiquitin molecules onto targeted proteins is a modification that can be reversed. This reversal is

called deubiquitination and is executed by proteases termed deubiquitinases (DUBs)<sup>[17]</sup>. DUBs specifically cleave ubiquitin after the terminal carboxyl group of ubiquitin and play a pivotal role in maintaining ubiquitin homeostasis<sup>[18,19]</sup>. Many DUBs have been shown to interact with E3 ligases, which suggests that a major function of DUBs is to control the stability of E3 ligases and subsequently destabilise the substrates of the cognate E3 ligase<sup>[20]</sup>.

## THE ROLE OF E3 UBIQUITIN LIGASES IN THE UPS PATHWAY

E3 ubiquitin ligases are often found overexpressed in a variety of human cancers, including lung cancer, and their deregulation has been shown to contribute to cancer development. As a result, increased attention is being paid to these E3 ubiquitin ligases and whether they can serve as potential anti-cancer targets. In targeted therapy, an ideal anti-cancer target should not only be overexpressed, but should meet additional criteria, such as its overexpression should be associated with poor prognosis, it plays a pivotal role in cancer genesis, inhibition induces apoptosis or growth reduction in the cancer cells, it is a “druggable” target (enzyme or cell surface molecule) that can be easily targeted, and finally, it is not expressed or is expressed at a very low level in normal cells.

E3 ubiquitin ligases can be divided in two major classes: the first class contains a C-terminal region Homologous to the E6-associated protein (E6-AP) carboxyl terminus (HECT), with an evolutionarily conserved cysteine residue required for the formation of a thiolester linkage with ubiquitin<sup>[21,22]</sup>. There are approximately 30 proteins containing the HECT domain. The second and largest class comprises E3 ubiquitin ligases that contain the really interesting new gene (RING) finger domain<sup>[23]</sup>. There are over 700 proteins containing the RING finger domain, but only a small part functions as an E3 ubiquitin ligase. Unlike RING proteins, most HECT proteins, if not all, are believed to function as E3 ubiquitin ligases. RING and HECT E3 ubiquitin ligases use different catalytic mechanisms to promote the transfer of ubiquitin to targeted substrates. RING E3 ubiquitin ligases can promote the direct transfer of ubiquitin from E2 to the targeted substrate, whereas HECT E3 ubiquitin ligases interact with the cognate E2, followed by the formation of a thiolester linkage with ubiquitin and subsequent transfer of ubiquitin to the targeted substrate (Figure 1).

The conjugation of one ubiquitin molecule to a protein is referred to as monoubiquitination, a process involved in protein trafficking, histone regulation, retrovirus budding and direct modulation of protein function<sup>[24]</sup>. As mentioned above, ubiquitin is attached to a lysine residue on the targeted substrate, however, ubiquitin itself also contains lysine residues that serve as self-conjugation sites. As a result, a chain of multiple ubiquitin molecules can be formed and appended to the targeted protein, which is referred to as polyubiquitination. Although monoubiquitination has been shown to be sufficient for the degra-

**Table 1** A list of E3 ubiquitin ligases that have been found deregulated in lung cancer, along with their substrate(s) (when known) and the processes in which they are involved related to lung cancer, with corresponding references

Process	E3 ubiquitin ligase	Substrate	Ref.
Cell proliferation	c-Cbl	EGFR	61, 62
	Nedd4	PTEN	65
	Siah2	HIPK2	56, 128
Cell cycle regulation	APC	OLC1	37, 68
	Cul3-based ligase	Rho GTPase; Rho BTB2	96
	CCNB1IP1	Cyclin B	93
	Parkin	Parkin, CDCrel-1	86, 88
	SCF component: Fbxo7		79, 80
	SCF component: Skp2	CdK: p27, p21, p53	72, 73, 74
	MDM2	P53, pRb	108, 117, 118, 161
	Parkin		90, 91
	Pirh2	P53	115, 162
	SCF component: SAG	c-Jun	132, 134, 136, 137
Apoptosis	Siah2	HIPK2	128, 129
	Topors	P53	123
	TRAF2	RIP1	127, 163
	XIAP	XIAP, AIF	104
	Cul3-based ligase	IKBKb	140
	FANCL	FANCD2	143-145

APC: Anaphase promoting complex; FANCL and FANCD2: Fanconi Anemia Complementation group type L and D2. HIPK2: Homeodomain-interacting protein kinase-2; SAG: Sensitive to Apoptosis Gene; OLC1: overexpressed in lung cancer 1; SCF: Skp, cullin, F-box protein; XIAP: X-linked inhibitors of apoptosis.

dation of some proteins, polyubiquitination accelerates the degradation of most proteins<sup>[25-27]</sup>. Self-conjugation of ubiquitin can occur through different lysine residues and it has been shown that polyubiquitin chains resulting from different ubiquitin linkages have distinct functions. Linkage through lysine-48 is the primary target signal for proteasomal degradation<sup>[28]</sup>, whereas ubiquitin chains linked through lysine-63 execute many functions including protein synthesis<sup>[29]</sup>, kinase activation<sup>[30]</sup> and DNA repair<sup>[31]</sup>. In addition, linkage through other lysine residues has been suggested including the involvement of lysine-6 linkage in the regulation of DNA repair<sup>[32]</sup>. Furthermore, linkage through lysine-29 has been shown to be involved in protein degradation, however, its function is not similar to that of linkage via lysine-48<sup>[33]</sup>.

E3 ubiquitin ligases can execute their function as a single peptide or they can act as multi-component complexes that function as RING-finger type E3 ubiquitin ligases. The distinct superfamily of E3 ubiquitin ligase complexes consists of the skp, cullin, F-box protein (SCF) family, the anaphase-promoting complex (APC) family, and the VHL-elongin C/elongin B (VCB) family<sup>[34]</sup>. The SCF family makes use of adaptor subunits called F-box pro-

teins that control substrate recognition through distinct protein-protein interaction domains<sup>[35]</sup>, whereas the APC family uses different adaptors and targets proteins that regulate mitosis<sup>[36]</sup>. The APC complex is composed of at least 10 subunits including yeast Apc2 and Apc11p<sup>[37]</sup>, which are thought to show homology to subunits of the SCF complex<sup>[38]</sup>. The VCB-like complexes possess a similar architectural structure as the SCF and APC family and on that basis are referred to be E3 ubiquitin ligases.

## LUNG CANCER

Lung cancer is the most commonly diagnosed cancer as well as the most common cause of cancer related deaths worldwide, and can be divided in small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC)<sup>[39,40]</sup>. The major problem in the treatment of lung cancer is the emergence of intrinsic and acquired drug resistance<sup>[41]</sup>. Despite increased knowledge on the molecular mechanisms contributing to drug resistance and the development of novel agents, the overall 5-year survival of patients diagnosed with lung cancer is less than 15%. This highlights the relevance and necessity of novel agents that can be used in combinational therapies to circumvent drug resistance. One of the strategies that is currently being investigated is targeting components of the UPS system.

In lung cancer, the deregulation of various UPS components has been observed<sup>[2,42]</sup>. For instance, the mRNA expression of E1-like ubiquitin-activating enzyme (UBE1L) is often reduced in lung cancer cells<sup>[43,44]</sup>. UBE1L conjugates IFN-stimulated gene, 15-kDa protein (ISG15) and was shown to promote a complex between ISG15 and cyclin D1, which results in cyclin D1 inhibition and subsequent lung cancer growth suppression<sup>[45]</sup>. In addition, mRNA expression levels of E2 ligases UBE2C and UBE2T were found to be significantly up-regulated in lung cancer tissues relative to normal lung tissues<sup>[46,47]</sup>, whereas mRNA expression of E2 ligase Hrad6B was found to be significantly decreased<sup>[48]</sup>. These E2 ligases are involved in multiple biological processes: UBE2C (also known as UBCH10) initiates the degradation of APC/cyclosome (APC/C) substrates thereby regulating progression through mitosis<sup>[49]</sup>, while UBE2T exerts its function in the fanconi anemia pathway by promoting monoubiquitination of the FANCD2 protein-a key step for efficient DNA repair<sup>[50]</sup>. In addition, Hrad6B is involved in UV mutagenesis, DNA repair<sup>[48,51]</sup> and was shown to be involved in histone methylation by promoting the ubiquitination of histone H2B<sup>[52]</sup>.

In this review, we provide an overview of E3 ubiquitin ligases that have been found to be deregulated in lung cancer and a few of them meet some of the criteria of being an ideal anti-cancer target (Table 1). Furthermore, we will discuss the biological processes in which these E3 ubiquitin ligases are involved related to lung cancer as well as their potency to function as “druggable” targets for the treatment of lung cancer.



## CELL PROLIFERATION

Tumorigenesis requires abnormal cellular proliferation. Consequently, signalling pathways controlling this complex process are the subjects of intensive research efforts. The RAS/MAPK pathway is one of the best-characterized signal transduction pathways involved in cellular proliferation. The GTPase RAS transmits extracellular signals from receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), to downstream effector proteins (*e.g.*, RAF, MEK and ERK) that besides cell proliferation also control differentiation, survival and apoptosis<sup>[53,54]</sup>. In *Drosophila*, the RING E3 ubiquitin ligase SINA was shown to be a critical component in RAS signalling located most downstream in the pathway<sup>[55]</sup>. SINA has two human homologs: SIAH1 and SIAH2 that share 76% and 68% sequence similarity, respectively<sup>[56]</sup>. Interestingly, SIAH2 protein levels were increased in highly aggressive lung tumors compared to little or no expression in normal lung tissues<sup>[57]</sup>. Moreover, similar levels of SIAH2 mRNA transcripts were detected in multiple lung carcinoma cell lines. However, SIAH2 expression is not restricted to tumorigenic cells but is also expressed by cells lacking any tumorigenic potential suggesting SIAH2 to be involved in all human proliferative cells<sup>[58]</sup>. As expected, inhibition of SIAH2 suppressed proliferation and reduced the tumorigenesis of human lung cancer cells. Recently, researchers identified the SIAH2-specific substrate homeodomain-interacting protein kinase-2 (HIPK2) which is a serine/threonine kinase that promotes p53-regulated gene expression by phosphorylating p53 at serine 46<sup>[23]</sup>. They found that overexpression of HIPK2 in lung cancer cells reduced cellular proliferation. This is in line with the suggestion that HIPK2 is tightly regulated in a p53-dependent manner in order to prevent ERK-mediated cell proliferation in the presence of activated p53<sup>[59,60]</sup>.

The RING E3 ubiquitin ligase c-Cbl is known for its function in cell signalling and protein ubiquitination of multiple substrates including EGFR<sup>[61]</sup>. By targeting EGFR for proteasomal degradation, c-Cbl negatively regulates EGF signalling and opposes cellular proliferation<sup>[62]</sup>. Recently, Tan *et al.*<sup>[42]</sup> determined the genetic variation and functionality of c-Cbl in NSCLC. They found a significant loss of heterozygosity of the c-Cbl locus in tumor samples from lung cancer patients compared to normal lung tissues. In addition, they identified novel somatic missense mutations of c-Cbl in multiple regions of the protein including the catalytic RING finger domain and the N-terminal tyrosine kinase binding (TKB) domain, both of which are vital for its E3 ubiquitin ligase activity<sup>[63,64]</sup>. Furthermore, overexpression of these mutations in NSCLC cell lines resulted in enhanced proliferative potential and cell motility suggesting an essential role for c-Cbl in lung tumorigenesis and metastasis.

In NSCLC, loss of the PTEN tumor suppressor is frequently observed leading to constitutive activation of the AKT pathway which is involved in fundamental

cellular processes including protein synthesis, cell proliferation and survival. Recently, the protein Neural precursor cell Expressed Developmentally Down-regulated 4-1 (Nedd4-1) was identified as the E3 ubiquitin ligase responsible for PTEN proteasomal degradation<sup>[65]</sup>. An additional study showed that Nedd4-1 is overexpressed in 80% of NSCLC tumors which correlates with the loss of PTEN protein<sup>[66]</sup>. Accordingly, knock-down of Nedd4-1 stabilized PTEN protein levels and, in addition, significantly reduced proliferation of NSCLC cells *in vitro* and tumor growth *in vivo*.

## CELL CYCLE REGULATION

In order for a multicellular organism to develop normally, tight regulation of the cell cycle is required<sup>[67]</sup>. Key regulators of the cell cycle are cyclins, which bind and activate cyclin-dependent kinases (CDKs) resulting in cell cycle progression. The cell cycle consists of four distinct phases: G<sub>1</sub>, DNA synthesis (S phase), G<sub>2</sub> and mitosis (M phase), with G<sub>1</sub> and G<sub>2</sub> functioning as “gap” phases separating S phase and M phase in time. Cells exit from mitosis upon degradation of mitotic cyclins, a process controlled by the RING E3 ubiquitin ligase anaphase-promoting complex (APC)<sup>[37]</sup>. As mentioned earlier, the APC complex consists of at least 10 subunits<sup>[37]</sup>. Among these subunits are the activator proteins cell-division cycle protein 20 (CDC20) and cadherin-1 (Cdh1), which regulate the activity and substrate specificity of APC. The E3 ubiquitin ligase APC, together with its regulatory subunits CDC20 and Cdh1, were found to be accountable for the degradation of the overexpressed in lung cancer 1 (OLC1) protein<sup>[68]</sup>. OLC1 is highly expressed in lung cancer tissues from patients with a history of cigarette smoking<sup>[68,69]</sup>. OLC1 degradation by the E3 ubiquitin ligase APC was compromised upon introducing cigarette smoke condensate (CSC). Several studies have revealed that OLC1 is involved in cytokinesis, a process following mitosis<sup>[70,71]</sup>. However, additional studies are required to clarify the exact role of OLC1 in lung tumorigenesis.

Another important regulator of cell cycle progression is the F-box protein Skp2. Skp2 is part of an SCF complex that targets cyclin-dependent kinase inhibitors p27, p21 and p57 for proteasomal degradation, thereby promoting G<sub>1</sub> to S phase transition<sup>[72-74]</sup>. Overexpression of Skp2 is frequently observed in lung cancer tissues and is associated with the invasive and metastatic potential of NSCLC cells<sup>[75,76]</sup>. Accordingly, several studies have demonstrated that inhibition of Skp2 suppresses the growth of lung cancer cells<sup>[5,77,78]</sup>.

The F-box protein Fbxo7 is also a component of an SCF complex and was found to selectively enhance CDK6 thereby regulating cell cycle progression<sup>[79,80]</sup>. Fbxo7 was reported to be upregulated in human lung cancers and to have transforming activity through cdk6<sup>[80]</sup>. Surprisingly, Fbxo7 does not seem to increase the degradation of the proteins with which it interacts

but rather increases their assembly and activity. However, novel Fbxo7-interacting proteins have been identified and are currently being investigated as candidates for Fbxo7-mediated ubiquitination.

In an attempt to identify novel tumor suppressors, Cesari and colleagues identified increased mRNA levels of parkin in NSCLC cells<sup>[81]</sup>. Conversely, a different study revealed a loss of parkin transcripts in NSCLC tumor tissues and showed that parkin expression was able to inhibit tumorigenicity in mice<sup>[82]</sup>. The parkin gene is mainly studied due to its pivotal role in the onset of autosomal recessive juvenile parkinsonism (ARJP)<sup>[83]</sup>. The parkin protein contains a RING finger motif and an ubiquitin-like domain, and many alternatively spliced isoforms have been identified<sup>[84,85]</sup>. Interestingly, parkin has been shown to exhibit E3 ubiquitin ligase activity targeting itself<sup>[86]</sup> and several other substrates for proteasomal degradation thereby regulating apoptosis and cell cycle<sup>[87-91]</sup>. However, the exact role of parkin in lung tumorigenesis needs to be further elucidated.

In contrast with studies on Skp2 and Fbxo7, researchers found that low levels of the E3 ubiquitin ligase CCNB1IP1 in NSCLC correlates with a lower overall survival<sup>[92]</sup>. CCNB1IP1 contains a RING finger domain and regulates cell cycle by interacting with cyclin B and promoting its degradation<sup>[93]</sup>. The exact role of CCNB1IP1 in lung tumorigenesis is not known.

A major class of ubiquitin ligases are the Cullin-based E3 ubiquitin ligases, which are incorporated in the SCF and APC complexes<sup>[94]</sup>. In mammals, eight distinct cullin proteins have been identified; Cul1 to Cul7 and PARC<sup>[95]</sup>. The Cul1-based E3 ubiquitin ligases are the best characterized and have been shown to control the protein levels of tumor suppressors and oncogenes, and are involved in cell cycle regulation<sup>[94]</sup>. The Cul3-based E3 ubiquitin ligases have recently emerged as key regulators of mitosis<sup>[96]</sup>. The atypical Rho GTPase RhoBTB2 is one of the substrates of Cul3-based E3 ubiquitin ligase complexes and its gene expression is ablated in 50% of lung cancer cell lines<sup>[97]</sup>. It has been suggested that RhoBTB2 functions as a tumor suppressor by recruiting proteins to a Cul3 ubiquitin ligase complex for degradation. However, it is unknown whether the ablation of RhoBTB2 in lung cancer cells correlates with deregulated levels of the Cul3 ubiquitin ligase.

## APOPTOSIS

The ability of cells to undergo apoptosis is vital for tissue homeostasis and development<sup>[98]</sup>. An essential step in apoptosis is the activation of caspases, a family of cysteine proteases<sup>[99]</sup>. The inhibitors of apoptosis (IAP) proteins are a family that negatively regulate caspases, with the X-linked IAP (XIAP) protein as the best-studied member<sup>[100-103]</sup>. XIAP contains a RING finger domain and has been characterized as an E3 ubiquitin ligase<sup>[104]</sup>. Surprisingly, it was observed that high levels of XIAP correlate with a significant longer overall sur-

vival of NSCLC patients and is suggested to associate with less aggressive NSCLC<sup>[105]</sup>. These observations are conflicting with a study in leukemia patients where they demonstrate a correlation between XIAP expression and a decreased overall survival<sup>[106]</sup>. This implies alternate functions of XIAP in different types of cancer.

In response to physiological stress, the p53 protein is activated and promotes either apoptotic cell death or cell arrest<sup>[107]</sup>. The levels of p53 are tightly regulated by the E3 ubiquitin ligase MDM2 through an auto-regulatory negative feedback loop; a p53-regulated gene induces MDM2 expression while MDM2 targets p53 for degradation by the 26S proteasome thereby controlling p53-mediated biological responses<sup>[108]</sup>. MDM2 is often overexpressed in several human cancers including lung cancer<sup>[109]</sup>. It has been shown that protein expression levels of MDM2 are overexpressed in 70% of NSCLC tissues compared to adjacent normal lung tissues<sup>[110,111]</sup>. Recently, a single nucleotide polymorphism-SNP309-was identified in the promoter region of MDM2 and was shown to induce MDM2 overexpression thereby influencing p53 activity<sup>[112]</sup>. Interestingly, a subsequent study revealed an association between SNP309 and increased NSCLC risk, which was predominantly seen among women<sup>[113]</sup>.

The E3 ubiquitin ligase Pirh2 is another protein that promotes p53 degradation<sup>[114]</sup>. The *Pirh2* gene is regulated by p53 and encodes a RING-finger containing protein that exerts the ubiquitination of p53 independently of MDM2. A study from Duan and colleagues showed that Pirh2 is overexpressed in the majority of lung cancer tissues when compared to normal lung tissues<sup>[115,116]</sup>. Furthermore, they found enhanced p53 ubiquitination which subsequently resulted in lower p53 expression in mouse lung tumors than in normal tissues. These results are consistent with their hypothesis that increased Pirh2 expression affects lung tumorigenesis by reducing p53 activity.

In addition to targeting p53 for proteasomal degradation, MDM2 has been shown to ubiquitinate the retinoblastoma protein (pRB) which plays a dual role in both apoptosis and cell proliferation<sup>[117,118]</sup>. Miwa and colleagues found that high expression levels of MDM2 correlated with low expression levels of pRB in a subset of NSCLC patients<sup>[117,119]</sup>. This correlation was mainly observed in NSCLC cells lacking wild-type p53. Miwa and co-workers suggest that MDM2-induced ubiquitination of pRB perturbs the pRB pathway and subsequently promotes carcinogenesis in a p53-independent manner.

Another protein that has been shown to interact with p53 is the RING finger protein topoisomerase I-binding protein (topors)<sup>[120-122]</sup>. This interaction results in p53 stabilization and consequent induction of either apoptosis or cell cycle arrest. Conversely, topors has been shown to possess E3 ubiquitin ligase activity targeting p53 for proteasomal degradation, although to a lesser extent than MDM2<sup>[123]</sup>. This insinuates that topors-induced p53 regulation does not only occur through ubiquitination but also by other mechanisms. Interestingly, preliminary studies have revealed an increase of the human topors

gene (also known as LUN) in various lung cancer cell lines<sup>[122,124]</sup>. Furthermore, expression of LUN was slightly downregulated along with progression of primary NSCLC tumors and strongly downregulated in nodal metastasis<sup>[124]</sup>. It is suggested that LUN might play a role in inhibition of nodal metastasis as well as the oncogenesis of NSCLC.

Besides the well known caspase inhibitor XIAP and E3 ubiquitin ligases that interact with p53 there are more E3 ubiquitin ligases alternatively expressed in lung cancer that are involved in the apoptotic pathway. For example, the E3 ubiquitin ligase Tumor necrosis factor receptor-associated 2 (TRAF2) was identified as a candidate radiosensitizing target in lung cancer<sup>[125]</sup>. TRAF2 belongs to a family of seven TRAF members (TRAF1-7) that play a role in a variety of biological processes including immunity, inflammation and apoptosis<sup>[126,127]</sup>. The TRAF2 protein contains a RING finger domain and mediates several signalling pathways involved in apoptosis protection<sup>[127]</sup>. It was found that TRAF2 is overexpressed in both lung carcinoma tissues and lung cancer cell lines<sup>[125]</sup>. In addition, downregulation of TRAF2 in radioresistant lung cancer cells caused growth suppression and radiosensitization, suggesting that TRAF2 may be an attractive drug target for anticancer therapy and radiosensitization.

Moreover, the RING E3 ubiquitin ligase SIAH2 has been shown to play a role in apoptosis. Activity of the recently identified pro-apoptotic SIAH2-specific substrate HIPK2 is considered to play a role in restraining tumor development by targeting tumor cells toward apoptosis upon genotoxic stress<sup>[60]</sup>. Inhibition of HIPK2 in lung cancer cells resulted in protection against UV-induced apoptosis<sup>[128,129]</sup>. In accordance, overexpression of HIPK2 sensitized lung cancer cells to UV-induced apoptosis and reduced cellular proliferation.

Finally, it has been shown that the Sensitive to Apoptosis Gene (SAG) is significantly overexpressed in NSCLC tumor tissues compared to adjacent normal lung tissues<sup>[130,131]</sup>. The SAG protein contains a RING finger domain and has been characterized as a component of SCF E3 ubiquitin ligases targeting several substrates for proteasomal degradation<sup>[132,133]</sup>. Interestingly, high mRNA levels of SAG correlate with poor survival of NSCLC patients suggesting SAG as a potential prognostic marker in NSCLC. Furthermore, inhibition of SAG sensitizes radioresistant NSCLC cells to ionizing radiation<sup>[131]</sup>. Under stress conditions, SAG has been shown to function as a proliferating factor that inhibits apoptosis and promotes cell growth<sup>[134-137]</sup>.

## GENE REGULATION

The nuclear factor- $\kappa$ B (NF- $\kappa$ B) is a key transcription factor that is thought to play a major role in carcinogenesis<sup>[138]</sup>. NF- $\kappa$ B controls genes that regulate a variety of biological processes including inflammation, innate and adaptive immunity, and stress responses. In lung

cancer, NF- $\kappa$ B is frequently expressed and was found to be involved in the pathogenesis of lung cancer<sup>[139]</sup>. The inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta (IKBKB) activates NF- $\kappa$ B and is a substrate of a Cul3 ubiquitin ligase complex. Recently, it was shown that genetic disruption of components of a Cul3 ubiquitin ligase complex results in elevated IKBKB levels and represents a mechanism of NF- $\kappa$ B activation in NSCLC<sup>[140]</sup>. Furthermore, inhibition of NF- $\kappa$ B was earlier shown to sensitize NSCLC cells to chemotherapy-induced apoptosis suggesting a possible role for the inactivation of NF- $\kappa$ B-induced pathways in the treatment of lung cancer<sup>[141]</sup>.

## DNA REPAIR

Upon DNA lesions, DNA damage surveillance systems are triggered and subsequently promote the activation of a multitude of genome-protection pathways<sup>[142]</sup>. One of these pathways involves the fanconi anemia (FA) proteins that are found to form a multi-protein complex that functions as an E3 ubiquitin ligase<sup>[143]</sup>. This E3 ubiquitin ligase complex exerts its function by monoubiquitinating FANCD2 during DNA replication or following DNA damage, mainly triggered by DNA crosslinking agents such as mitomycin C or Cisplatin<sup>[143]</sup>. Monoubiquitinated FANCD2 can interact with FANCD1/BRCA2 and others to repair damaged DNA. It was found that the lung cancer cell line Calu-6 harbors an impaired FA-BRCA pathway resulting from alternatively expressed FANCL, a catalytic subunit of the E3 ubiquitin ligase complex<sup>[144,145]</sup>. The pathway integrity was re-established upon FANCL complementation and reduced the hypersensitivity of Calu-6 cells to mitomycin<sup>[145]</sup>. Based on these results, it is suggested that the status of the FA-BRCA pathway could play an important role in determining the sensitivity of cancer cells to DNA crosslinking agents.

## OTHERS

Most of the E3 ubiquitin ligases described above are also involved in other biological processes. For example, besides its involvement in cell proliferation and apoptosis, Siah2 plays a role in the physiological responses to hypoxia and was shown to target a rate-limiting enzyme in the mitochondrial Krebs cycle<sup>[146,147]</sup>. In addition, c-Cbl is involved in cell proliferation but also plays a critical role in angiogenesis and is involved in immunity by targeting many protein substrates for proteasomal degradation<sup>[148-151]</sup>. However, at present the E3 ubiquitin ligases that have been found to be deregulated in lung cancer have not been described to be involved in biological processes other than the ones we have discussed in this review.

## CONCLUSION AND PERSPECTIVES

Currently, the major issue in targeting E3 ubiquitin ligases is the lack of specific inhibitors in clinical trials. Over



the past years, many research efforts have focused on the development of proteasome inhibitors. At present, Bortezomib is the only selective and reversible proteasome inhibitor approved by the United States Food and Drug Administration (FDA) and the European Medicine Agency and it is being used for the treatment of relapsed/refractory multiple myeloma and mantle cell lymphoma<sup>[152,153]</sup>. However, its induced cytotoxicity is based on overall inhibition of proteolysis of many cellular proteins. By selectively inhibiting an E3 ubiquitin ligase the proteins are stabilized that are regulated by this E3 ubiquitin ligase and thereby circumvent undesired effects on other cellular proteins. The deregulation of E3 ubiquitin ligases has been shown to contribute to cancer development and they are often found overexpressed in lung cancer<sup>[4,5]</sup>. Altogether, targeting E3 ubiquitin ligases has gained increasing attention, which has led to the development of high-throughput screening assays to identify inhibitors of multiple E3 ubiquitin ligases<sup>[154,155]</sup>. For example, small molecule inhibitors of the E3 ubiquitin ligase MDM2 have been identified and developed such as cis-imidazolines, benzodiazepines and spiro-oxindoles<sup>[156,157]</sup>. These inhibitors selectively inhibit MDM2 E3 ubiquitin ligase driven polyubiquitination of p53 with barely any effect on other enzymes using ubiquitin. However, one major concern is the selectivity between normal and cancer cells. Although it is still unclear, the activation of p53 by these MDM2 inhibitors in normal cells induces growth arrest rather than apoptosis making it achievable to obtain a therapeutic window<sup>[158]</sup>. Excitingly, inhibitors targeting MDM2 are now in clinical trials and pave the way for novel treatment strategies for cancer patients including those diagnosed with lung cancer<sup>[157]</sup>. Eventually, these inhibitors can be utilized in combination with other therapies, *e.g.*, chemotherapy in order to circumvent drug resistance which is an important problem in the treatment of patients with lung cancer.

In addition to MDM2, there are more E3 ubiquitin ligases that meet some of the criteria of being an ideal anti-cancer target. For example, the HECT E3 ubiquitin ligase Nedd4-1 is overexpressed in the majority of NSCLC tumors and its inhibition reduces the proliferation of NSCLC cells<sup>[60]</sup>. Furthermore, the SCF component SAG is frequently overexpressed in NSCLC tissues<sup>[131]</sup>. Importantly, high SAG expression is correlated with poor survival of NSCLC patients and could be a useful prognostic marker. In addition, other SCF components have been described in lung cancer. For example, the F-box protein Skp2 is often overexpressed in lung cancer tissues and is associated with the metastatic and invasive potential of NSCLC cells<sup>[75,76]</sup>. However, targeting SAG or Skp2 is challenging, since they are part of an SCF complex containing multiple components. Therefore, a general inhibitor against SAG or Skp2 may not have the desirable specificity against any SCF complex. More ideal anti-cancer targets would be the RING E3 ubiquitin ligase Pirh2 and TRAF2. Pirh2 is often overexpressed in many cancer tissues including lung cancer<sup>[139]</sup>.

In addition, TRAF2 is overexpressed in the majority of lung cancer tissues and its downregulation suppresses cell growth and sensitizes otherwise radioresistant lung cancer cells. However, there are no inhibitors targeting these E3 ubiquitin ligases that are currently being tested in clinical trials.

Like E3 ligases, DUBs can be considered as potential anti-cancer targets. Although DUB inhibitors or activators have yet to successfully enter the clinic, multiple DUBs have been implicated in neoplastic disease such as ubiquitin-specific protease 4 (USP4), USP6, and USP8<sup>[160]</sup>.

Although the biological functions of many E3 ubiquitin ligases are still not fully understood, it has become clear that some E3 ubiquitin ligases are promising anti-cancer targets. Despite the fact that we are still facing issues such as selectivity between normal and cancer cells and specificity between E3 ubiquitin ligase and protein substrates, the approval of Bortezomib and the recent entry of MDM2 inhibitors into clinical trials will further stimulate the development of specific E3 ubiquitin ligase inhibitors for the treatment of many cancers including lung cancer.

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