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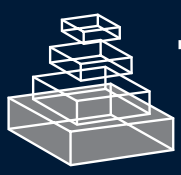
THE KEY ROLE OF UBIQUITINATION AND SUMOYLATION IN SIGNALING AND CANCER

Topic Editors
Hui-Kuan Lin and Wenyi Wei



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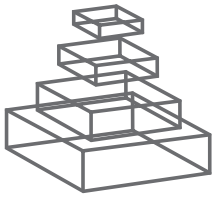

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THE KEY ROLE OF UBIQUITINATION AND SUMOYLATION IN SIGNALING AND CANCER

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Ubiquitination and Sumoylation are two important posttranslational modifications that play pivotal roles in a variety of biological functions.

Although Ubiquitination is traditionally viewed as a critical mark targeting proteins for proteasome-dependent degradation, recent studies reveal that it also plays nonproteolytic functions. In contrast, Sumoylation is long thought not to target proteins for degradation, accumulating evidence suggests that it can serve a priming effect prerequisite for ubiquitination, thereby inducing protein ubiquitination and degradation.

Thus, there is an important cross-talk between sumoylation and ubiquitination in determining protein fate. Deregulation in these two marks may cause aberrant activity of proteins and in turn contributes to cancer development.

In this Research Topic, we accept review articles, perspectives, research articles covering any one or both of these two posttranslational modifications in regulating diverse signal transduction pathways and providing the novel insights in unraveling the puzzle as to how they may regulate cancer progression and metastasis.

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EDITORIAL
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The key role of ubiquitination and sumoylation in signaling and cancer: a research topic

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INTRODUCTION

Ubiquitination and sumoylation are two important post-translational modifications that play pivotal roles in signaling regulation, protein trafficking, protein stability, and transcriptional regulation, ultimately regulating a plethora of biological processes such as cell survival, cell migration, DNA damage response (DDR), neurodegeneration, and cancer. Although ubiquitination is traditionally viewed as a critical mark targeting proteins for proteasome-dependent degradation, recent studies have revealed that it also has non-proteolytic functions. In contrast, sumoylation is long thought not to target proteins for degradation, but accumulating evidence suggests that it can serve as a priming pre-requisite for ubiquitination, thereby inducing protein ubiquitination and degradation. Thus, there is an important cross-talk between sumoylation and ubiquitination in determining protein fate. Deregulation in these two processes may cause aberrant activity of proteins and in turn contributes to cancer development. In this Research Topic, we assembled 10 review articles to discuss the role of these two post-translational modifications in regulating diverse signal transduction pathways, thereby providing novel insights in unraveling the puzzle as to how they may regulate cancer progression.

The ubiquitin-proteasome system has been recently characterized as a major regulatory mechanism for ensuring ordered and coordinated cell cycle progression by selective degradation of key cell cycle regulators. Two related, multi-subunit E3 ubiquitin ligase enzymes, the Anaphase Promoting Complex (APC) and the Skp1-Cullin1-F-box complex (SCF) are thought to be the major driving forces governing cell cycle progression. Nagi Ayad's group discussed the important physiological functions of APC/C as well as the upstream signaling pathway that governs the timely regulation of APC/C. Given the critical role of APC/C in cell proliferation and development, they also illustrated the emerging contribution of APC/C in tumorigenesis and proposed APC/C intervention as a potential anti-cancer therapeutic approach.

Among the SCF-type of E3 ubiquitin ligases, SCF^{Skp2} is one of the most well-studied E3 ligases and Skp2 overexpression is frequently observed in various types of human cancers including breast cancer. The Wei group recently summarized the oncogenic role of Skp2 in breast cancer development. The interplay between Skp2 and other major signaling pathways as well as the recent advances in identifying Skp2 downstream substrates were also discussed. Lastly, they proposed specific Skp2 inhibitors as novel anti-breast cancer agents.

Unlike Skp2, VHL forms a distinct type of E3 ligase by associating with Elongin B, Elongin C, and Cullin 2. The Yang group discussed the important role of VHL in hypoxia sensing and kidney disease development by targeting HIF for ubiquitination and destruction in a hydroxylation-dependent manner. They also summarized the recent advances in understanding the tumor suppressor role of VHL independent of HIF. The identification of novel VHL substrates including PKC and EGFR and their relevance to signaling and cancer development were also discussed.

In addition to associating with VHL, the Elongin B/C complex can also interact with the SOCS box-containing proteins and Cullin 2 or Cullin 5 to form distinct functional E3 ubiquitin ligases. The Takumi Kamura group contributed a comprehensive review that described the recent advances in further characterizing the assembly as well as the physiological functions of the Elongin B/C-containing E3 ligases, which are further divided into the Cullin 2-type and the Cullin 5-type. They also summarized the newly identified downstream substrates, which provided an important insight into the critical roles of Elongin B/C-containing E3 ligases in regulating a variety of cellular functions.

The Pengbo Zhou group summarized the recent progress in functional analysis of another major class of E3 ubiqutin ligases, the Cullin 4-containing (CRL4) family of ligases. In this review, the authors updated the recent understanding of the two Cullin 4 family members, Cullin 4A and Cullin 4B in human cancer and neuronal disease development. In addition, the recent advances in identifying novel substrates for various Cullin 4-containing E3 ligases that typically associate with a specific DDB1-Cullin 4 associated factor (DCAF) were further discussed. More importantly, given their critical roles in tumorigenesis, the authors speculated that Cullin 4A and Cullin 4B could be potentially pursued as new targets for cancer prevention.

Protein kinases such as Akt, MAPK, and IKK are commonly upregulated and/or activated in a variety of human cancers. The Hung group summarized recent advances of how these oncogenic kinases regulate protein ubiquitination and degradation. They proposed that protein phosphorylation induced by these kinases provides a scaffold for the recognition of ubiquitin ligases (E3s), thereby eliciting protein ubiquitination and degradation. Several oncogenic proteins, such as β-catenin, MCL-1, and Snail, known to be regulated by these oncogenic kinases, were also discussed.

In addition to kinase regulation, ubiquitination can also orchestrate DDR by controlling protein degradation and non-proteolytic pathways. DDR triggers serial signaling events that are involved in DNA damage repair, transcription regulation, growth arrest, and apoptosis. The Jin group discussed the role of Cullin/RING ubiquitination ligase 1 (CRL1) and CRL4 in multiple steps of the DDR by regulating protein stability. The Yan group further illustrated the role of histone ubiquitination and deubiquitination in regulating transcription and DDR. The E3 ligases and deubiquitinating enzymes regulating the histone ubiquitination and deubiquitination processes and DDR are discussed.

Like protein ubiquitination and phosphorylation, sumoylation is another important post-translational modification that is also critically involved in DDR. Upon DNA damage, sumoylation of proteins like 53BP1 and BRCA-1 involved in DDR and DNA repair is induced and may play a role in DDR by inducing its E3 ligase activity. David Ann and colleagues discussed the potential crosstalk between sumoylation and ubiquitination and speculated that RNF4 may be a critical E3 ligase that connects the sumoylation and ubiquitination crosstalk to participate in ATM-mediated signaling and DDR.

Although ubiquitination is traditionally viewed as a critical marker targeting proteins for proteasome-dependent degradation, recent studies reveal that it also plays non-proteolytic functions. K48-linked ubiquitination in general leads to protein degradation, while K63-linked ubiquitination fails to do so. The Lin group and colleagues focused on a new type of ubiquitination called K63-linked ubiquitination and proposed that K63-linked ubiquitination serves as an important activation mark for oncogenic kinase activation, thereby contributing to cancer development. Thus, targeting K63-linked ubiquitination may be employed for cancer therapy.

ACKNOWLEDGMENTS

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REVIEW ARTICLE
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K63-linked ubiquitination in kinase activation and cancer

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Ubiquitination has been demonstrated to play a pivotal role in multiple biological functions, which include cell growth, proliferation, apoptosis, DNA damage response, innate immune response, and neuronal degeneration. Although the role of ubiquitination in targeting proteins for proteasome-dependent degradation have been extensively studied and well-characterized, the critical non-proteolytic functions of ubiquitination, such as protein trafficking and kinase activation, involved in cell survival and cancer development, just start to emerge, In this review, we will summarize recent progresses in elucidating the non-proteolytic function of ubiquitination signaling in protein kinase activation and its implications in human cancers. The advancement in the understanding of the novel functions of ubiquitination in signal transduction pathways downstream of growth factor receptors may provide novel paradigms for the treatment of human cancers.

Keywords: protein kinase, ubiquitination, phosphorylation, Akt, TRAF6, NF-κB, tumorigenesis

INTRODUCTION

Posttranslational modification of cellular proteins, such as phosphorylation, ubiquitination, SUMOylation, PARylation, to name a few, have been shown to play a pivotal role in multiple cellular processes such as proliferation, apoptosis, senescence, autophagy, DNA damage repair, innate immune response, and neuron degeneration (Kim et al., 2008b; Kirkin et al., 2009; Kraft et al., 2010; Gao et al., 2011). Ubiquitination is originally described as a process by which ubiquitin is covalently attached to the lysine residue of a target protein for proteasome-mediated degradation by three enzymes, namely E1 (ubiquitin-activating enzyme), one of many E2s (ubiquitin-conjugating enzymes, UBCs), and one of many E3s (ubiquitin ligases; Bhoj and Chen, 2009; **Figure 1**). As ubiquitination is a reversible process, ubiquitin covalently attached to a target protein can be removed by deubiquitinating enzymes (DUBs; **Figure 1**). Thus the balance between the activities of ubiquitin enzymes and DUBs determines the level and activity of a target protein.

Although ubiquitination most often lead to proteasome-mediated degradation of the target protein (Pickart, 2001; Weissman et al., 2011), recent evidence suggests non-proteolytic functions for ubiquitination in the regulation of protein subcellular localization and activity (Giasson and Lee, 2003; Bhoj and Chen, 2009; Chen and Sun, 2009; Hoeller and Dikic, 2009; Raiborg and Stenmark, 2009; de Bie and Ciechanover, 2011). These novel findings suggest that posttranslational modification of protein by ubiquitin and ubiquitin-like proteins is involved in a much wider range of cellular processes and signal transduction pathways important for numerous biological functions.

In this review, we will focus on the recent advances in the novel role of ubiquitination in the NF-κB activation and PKB/Akt kinase activation and their implications in human cancers. In addition, we will discuss some potential therapeutic strategies for targeting the ubiquitination pathways in the treatment of human cancers.

UBIQUITIN–PROTEASOME SYSTEM

A large number of proteins are posttranslational modified by ubiquitin. Ubiquitin, a 76-amino acid protein, is highly evolutionarily conserved across species. In the ubiquitination process, ubiquitin is covalently conjugated to lysine residues of substrate proteins through a three-step enzymatic reaction. Ubiquitin is first activated by ubiquitin-activating enzyme (E1). The activated ubiquitin is then delivered to the ubiquitin-conjugating enzyme (E2), and will be transferred from the E2 to a lysine residue of a target protein by ubiquitin ligase (E3; **Figure 1B**).

As a general principle, the ubiquitin-conjugating enzyme E2 determines the type of ubiquitination modification to occur and the ubiquitin ligase E3 confers substrate specificity. Currently, there are two E1s, 50 E2s, and 600 E3s identified in human genome (Bhoj and Chen, 2009). There are seven lysine (K) residues (K6, K11, K27, K29, K33, K48, and K63) in the ubiquitin, which have been identified to be utilized for ubiquitination chains (**Figure 1A**). Most E2s trigger the K48-linked ubiquitination, in turn leading to proteasome-dependent degradation. However, the functions of ubiquitin conjugation are not limited to the ubiquitin–proteasome pathway. Mono and polyubiquitins are used as signals in various pathways including endocytosis, DNA repair, apoptosis, and transcriptional regulation. Polyubiquitin chains can be formed using lysine residues other than K48, the linkage required for proteasomal degradation (**Figure 1B**).

Ubiquitin-conjugating enzyme 13 is a major E2 that induces K63-linked ubiquitination with the assistance of its cofactor

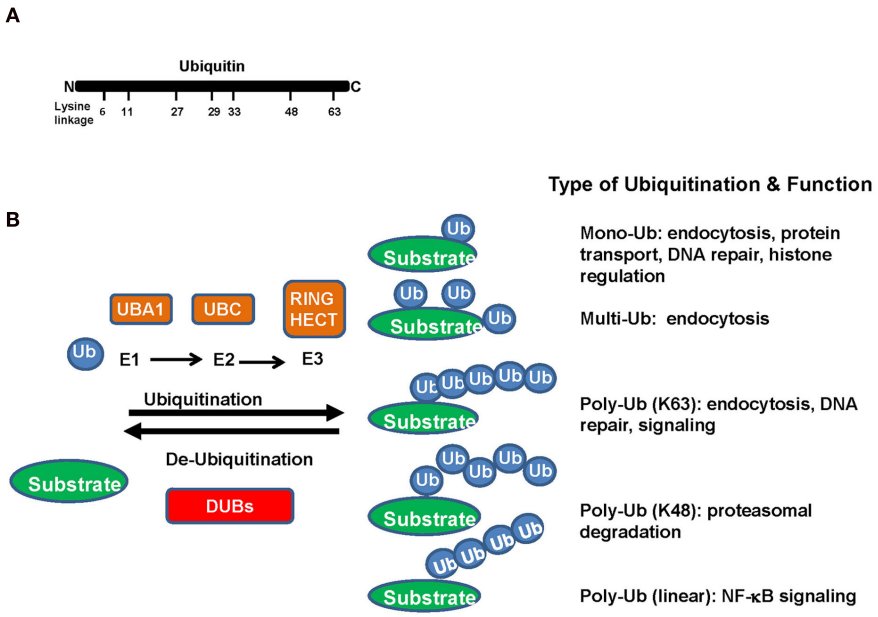

FIGURE 1 | (A) Ubiquitin, a 76-amino acid protein, is highly evolutionarily conserved across species. It contains seven lysine (K) residues (K6, K11, K27, K29, K33, K48, and K63) in the ubiquitin, which have been identified to be utilized for ubiquitination chains. **(B)** In the ubiquitination process, ubiquitin is covalently conjugated to lysine residues of substrate proteins through a three-step enzymatic reaction. Ubiquitin is first activated by ubiquitin-activating enzyme (E1). The activated ubiquitin is then delivered to the ubiquitin-conjugating enzyme (E2), which will be transferred from the E2 to a lysine residue of a target protein by ubiquitin ligase (E3). The function usefulness of ubiquitin conjugation is not limited to the ubiquitin–proteasome pathway. Mono- and polyubiquitins are used as signals in various pathways including endocytosis, DNA repair, apoptosis, and transcriptional regulation.

UEV1A. In stark contrast to the role of K48-linked ubiquitination in targeting its substrate protein for proteasome-degradation, the K63-linked ubiquitination does not induce proteasome-dependent degradation, but serves as a molecular platform for protein/protein interaction important for kinase signaling activation, receptor endocytosis, protein trafficking, and DNA damage repair. Although other types of ubiquitination modifications are also observed, the exact role that they play is unclear. Recent studies suggest that they can be involved in protein degradation or non-proteolytic functions (**Figure 1B**; Adhikari and Chen, 2009; Heemers and Tindall, 2009; Xu et al., 2009).

The E3s fall into two major families: E3s with HECT (homologous to the E6-AP carboxyl terminus) domain and E3s with RING (a really interesting new gene) or RING-like domain (e.g., U-Box and PHD domain; Bhoj and Chen, 2009). There are ∼600 RING finger and ∼30 HECT ligases in the human genome. Most of E3s recognize substrate proteins for K48-linked ubiquitination. However, several E3s can target proteins for K63-linked ubiquitination, such as HectH9, Mdm2, TNF receptor-associated factor 6 (TRAF6; tumor necrosis factor receptor-associated factor 6), cIAP1/2 (cellular inhibitor of apoptosis protein 1/2), CHIP, Parkin, UCHL1, TRAF2, ITCH, and NEDD4-2 (Adhikary et al., 2005; Huen et al., 2007; Mailand et al., 2007; Bertrand et al., 2008; Bhoj and Chen, 2009; Lim and Lim, 2011). Interestingly, HectH9, Mdm2, RNF8 (ring finger protein 8), and cIAP1/2 can trigger both K63- and K48-linked ubiquitination. For example, HectH9 not only targets Myc for K63-linked ubiquitination, which is critical for Myc transcriptional activation and the expression of a subset of Myc target genes, but also target MCL-1 and $p19^{Arf}$ for ubiquitination-dependent degradation (Adhikary et al., 2005; Chen et al., 2005; Zhong et al., 2005). So far, TRAF6 and RNF168 are the only two known E3s that selectively target substrate proteins for K63-linked ubiquitination, and thus play an important role in the innate immune response, DNA damage response (DDR), and tumorigenesis (Huen et al., 2007; Mailand et al., 2007; Bhoj and Chen, 2009).

THE DEUBIQUITINATING ENZYMES

Protein ubiquitination is a reversible process, and it is tightly controlled at both the levels of ubiquitin-conjugation and ubiquitin-deconjugation. Removal of ubiquitin on proteins is carried out by the DUBs. There are at least 98 DUBs encoded in the human genome, which can subdivided into six subfamilies based on sequence and structural similarity: ubiquitin-specific proteases (USP), ubiquitin carboxyl-terminal hydrolases (UCH), ovarian tumor-like proteases (OTU), JAMM/MPN metalloproteases, Machado–Jakob-disease (MJD) proteases, and the recently discovered monocyte chemotactic protein-induced protein (MCPIP) family (Sun, 2008; Bhoj and Chen, 2009; Hoeller and Dikic, 2009; Liang et al., 2010; Fraile et al., 2011). One mechanism to control the activities of DUB is the substrate specificity, the type of ubiquitin-chain linkages that are processed. Structural and functional studies have suggested that USPs and OTUs recognize and remove either Lys48- or Lys63-linked polyubiquitin chains. For example, USP14 removes Lys48-linked chains (Hu et al., 2005), whereas CYLD only efficiently processes Lys63 linkages and linear ubiquitin chains (Komander et al., 2009). Some OTU family components such as

OTUB1 and A20 hydrolyze Lys48-linked chains, whereas TRABID and OTUD5 have preference for Lys63 linkages (Virdee et al., 2010). Furthermore, Cezanne, suppressesing NF-κB activation by targeting RIP1 for deubiquitination (Enesa et al., 2008), preferentially cleaves Lys11 over Lys48 and Lys63 linkages (Bremm et al., 2010), whereas JAMMs share the specificity for Lys63-linked polyubiquitin (McCullough et al., 2004; Sato et al., 2008; Cooper et al., 2009). Finally, the Josephin ATXN3-editing activity shows a restricted specificity for K63-linked chains (Winborn et al., 2008). The activity of DUBs regulates the turnover rate, activation, recycling, and localization of multiple proteins, and thus plays an essential role in cell homeostasis, protein stability, and a wide range of signaling pathways (**Table 1**) (Cohn et al., 2007; Shao et al., 2009; Lee et al., 2010; Nakada et al., 2010; Wiltshire et al., 2010). Importantly, deregulated DUB function has been linked to several diseases, including cancer (**Table 1**) (Nicassio et al., 2007; Bhoj and Chen, 2009; Novak et al., 2009; Fraile et al., 2011; Luise et al., 2011; Metzig et al., 2011).

EMERGING ROLES FOR K63-UBIQUITINATION IN NF-κB ACTIVATION, AND KINASE ACTIVATION

K63-UBIQUITINATION PLAYS AN IMPORTANT ROLE IN NF-κB ACTIVATION AND INFLAMMATION

Emerging evidence suggests that K63-linked ubiquitination also plays a pivotal role in the regulation of pathways that have been implicated in inflammatory response and cancer development, such as NF-κB (Skaug et al., 2009). The canonical NF-κB activation pathway is mediated through IKK, an upstream kinase responsible for IκB phosphorylation and subsequent degradation (**Figure 2**). IKK is consisted of IKKα kinase, IKKβ kinase, and IKKγ regulatory subunit (also known as NEMO; Chen, 2005; Bhoj and Chen, 2009; Skaug et al., 2009), which phosphorylates IκB and promotes SCF-βTrCP-mediated K48-linked ubiquitination and subsequent degradation by proteasome. Degradation of IκB results in NF-κB nuclear translocation and activation. In the non-canonical pathway, NIK-mediated-phosphorylation of IKKα results in its activation and subsequent phosphorylation of p100, which recruits a K48 E3 ligase to targetp100 for partial proteasomal degradation (Martinez-Forero et al., 2009; Skaug et al., 2009). A stop signal within the p100 sequence will terminate the proteasomal degradation process and the p52 transcription factor is then released and forms heterodimer NF-κB2 transcription factor with REL-B, which translocates to the nucleus and regulates gene expression. K63-linked ubiquitination has been shown to play an important role in both canonical and non-canonical NF-κB activation pathways (**Figure 2**).

In both canonical and non-canonical NF-κB pathways, the engagement of cell surface receptors, such as TNFR, IL-1R, Toll-like receptor (TLR), and CD40 by ligand stimulation, leads to the recruitment of the TRAFs such as TRAF2 and TRAF6, to the receptors (Skaug et al., 2009; **Figure 2**). TRAF proteins are ubiquitin ligases containing a highly conserved N-terminal RING domain except for TRAF1. TRAF6 has been demonstrated to play an important role in innate immune response and apoptosis by regulating TLR and TGF-β signaling involved in NF-κB activation and p38 activation, respectively (Chen, 2005; Sorrentino et al., 2008; Yamashita et al., 2008; Heldin et al., 2009; Skaug et al., 2009).

In the canonical pathways, after ligand binding to IL-1R or TLRs, the adaptor protein MyD88 is recruited to the cytoplasmic tail of the receptor and facilitates its interaction with IRAK4 and IRAK1 serine/threonine protein kinases. IRAK4 in turn phosphorylates and activate IRAK1 (Ordureau et al., 2008), which

Table 1 | Deubiquitinating enzymes (DUBs) play important roles in NF-κB activation, DNA damage response, and cancer.

DUBs	Substrates	Function	Role in cancer	References
A20	IKKγ/NEMO, RIP1, TRAF6, MALT1	Negatively regulates the NF-κB pathway	Tumor suppressor	Mauro et al. (2006), Sun (2008), Kato et al. (2009), Novak et al. (2009), Compagno et al. (2009), Bhoj and Chen (2009)
CYLD	IKKγ/NEMO TRAF2, RIP1, TAK1, BCl3	Negatively regulates the NF-κB pathway	Tumor suppressor	Brummelkamp et al. (2003), Kovalenko et al. (2003), Sun (2008), Sun (2010), Bhoj and Chen (2009)
Cezanne	RIP1	Negatively regulates the NF-κB pathway	Unknown	Enesa et al. (2008)
USP2	Unknown	Positively regulates the NF-κB pathway	Downregulated in breast cancer	Metzig et al. (2011)
USP3	H2A, H2B	Required for DSB repair	Unknown	Nicassio et al. (2007)
USP11	Unknown, IκBα	Regulates HR pathway; Negatively regulates NF-κB pathway	Overexpressed in malignant melanoma, associated with a more aggressive & invasive phenotype	Wiltshire et al. (2010), Luise et al. (2011)
BRCC36	γH2AX	Regulates DSB repair pathway	Unknown	Shao et al. (2009)
OTUB1	Unknown	Regulates DSB pathway by Inhibit RNF168 activity	Unknown	Nakada et al. (2010)
USP1	FANCI–FANCD2, PCNA	Negatively regulates ICL repair	Unknown	Cohn et al. (2007), Lee et al. (2010)

USP3, ubiquitin-specific peptidase 3; CYLD, cylindromatosis tumor suppressor; ICL, interstrand crosslink repair; BRCC36, BRAC1/BRAC2-containing complex subunit 36.

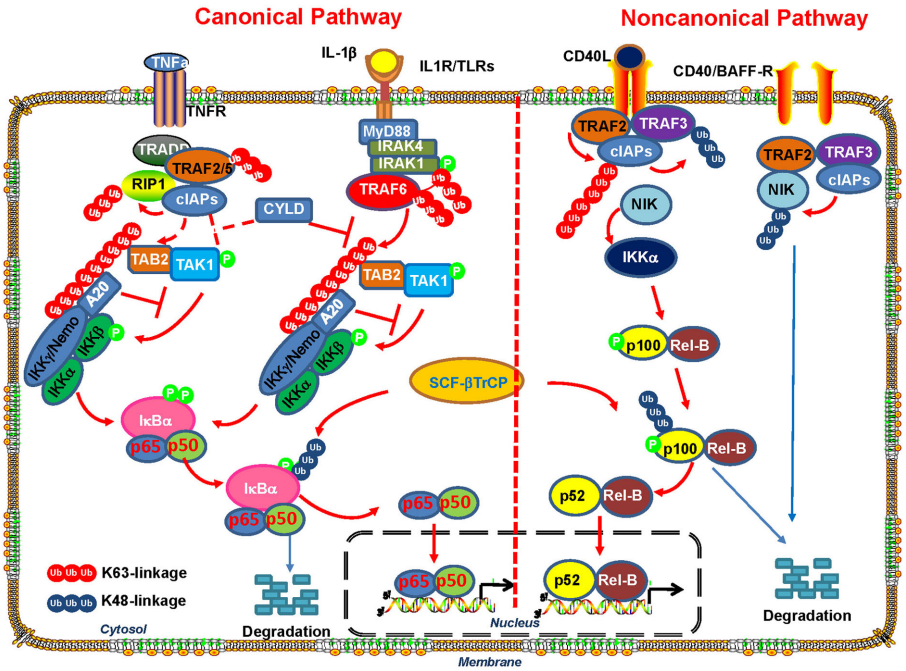

FIGURE 2 | The role of K63-linked polyubiquitination in the activation of canonical and non-canonical NF-κB signaling pathway. In both canonical and non-canonical NF-κB pathways, ligand binding to receptors results in the recruitment of the TNF receptor-associated factors (TRAFs) to the receptors. In the canonical pathway, the recruitment of E3 ligase TRAF6promotesits auto-polyubiquitinating and other neighboring target proteins via K63-linked polyubiquitin chains. Unanchored free K63-linked polyubiquitin then directly activate TAK1 kinase, which then phosphorylates and activates IKK-β to induce NF-κB activation. In the non-canonical pathway, TRAF3, TRAF2, and cIAP promote rapid proteasomal degradation of NIK by the ubiquitin–proteasome system under un-stimulated condition. Various stimuli release NIK from TRAF3 inhibition by triggering cIAP activation by TRAF2-mediated K63-polyubiquitination and subsequent degradation of TRAF3. NIK-mediated-phosphorylation of p100 and its subsequent processing promotes RelB/p52 nuclear translocation and NF-κB activation.

then recruits E3 ligase TRAF6 that functions together with dimeric ubiquitin-conjugating enzyme complex UBC13/UEV1A to induce its auto-polyubiquitinating and other neighboring target proteins via K63-linked polyubiquitin chains (Sorrentino et al., 2008; Yamashita et al., 2008; Martinez-Forero et al., 2009; Skaug et al., 2009). It has been proposed that K63-linked ubiquitination chains induced by TRAF6 serve as docking sites for TAB2, which contains ubiquitin-binding domains (UBD; Kanayama et al., 2004; Kulathu et al., 2009). TAB2 and TAB1 then promote the recruitment of the serine–threonine kinase TAK1 into the complex (Kanayama et al., 2004; Lynch and Gadina, 2004; Sun et al., 2004; Shambharkar et al., 2007) and its subsequent activation. Activated TAK1 then phosphorylates and activates IKK-β to promote IκB proteasomal degradation and translocation of NF-κB members p65 and p50 into the nucleus, in turn resulting in NF-κB activation (**Figure 2**). K63-linked polyubiquitination is critical in this process, because deletion of the UBAN domain of NEMO (IKKγ) abrogates IKK signaling. Although it was previously proposed that K63-linked polyubiquitination of several proteins in the IL-1LR/TLR pathways, such as TRAF6, IRAK1, and IKKγ/NEMO, are critical for the activation of TAK1 and IKK, a recent report provides compelling evidence that none of these polyubiquitin chains on these proteins are required for TAK1 activation (Xia et al., 2009). Instead, unanchored free K63-linked polyubiquitin chains, which are not conjugated to any target protein, directly activate TAK1 kinase through binding to TAB2 (Xia et al., 2009). Two possible mechanisms have been proposed for the unanchored polyubiquitin chains-mediated activation of TAK1 (Xia et al., 2009). One possible explanation is that TAK1 complex is brought into close proximity by the binding of TAB2 to the polyubiquitin chain, which will result in TAK1 autophosphorylation at Thr-187 and its activation. Another possibility is that polyubiquitin binding induces conformational change of TAK1 and thus allosterically activates the TAK1 complex. Free K63-linked polyubiquitin chains also directly bind to IKKγ/NEMO and activate IKK, which will lead to NF-κB activation (Xia et al., 2009).

TAK1 not only plays a role in NF-κB activation, but also in MAPK activation in response to numerous inflammatory stimuli (Wang et al., 2001; Skaug et al., 2009). TAK1 directly phosphorylates and activates MKK6 in an ubiquitin-dependent manner. In addition, knockdown of TAK1 by siRNA or TAK1 inhibitors suppresses IL-1β- and TNFα-induced IKK and c-Jun N-terminal kinase (JNK) activation (Ninomiya-Tsuji et al., 2003; Takaesu et al., 2003; Kanayama et al., 2004). Furthermore, inactivation of *Tak1* in *Drosophila melanogaster* (Vidal et al., 2001; Chen et al., 2002; Silverman et al., 2003), or deletion of *Tak1* in multiple mouse cell types abolishes IKK and MAPK activation by a variety of NF-κB agonists (Sato et al., 2005; Shim et al., 2005; Liu et al., 2006; Wan et al., 2006). Another study has further demonstrated a role for ubiquitination of TAK1 as a scaffold to recruit mitogen-activated

MEKK3 (protein kinase kinase kinase 3) and subsequently to activate the MAP kinase pathway (Yamazaki et al., 2009).

In addition to TAK1, other kinases also play a role in NF-κB activation. RICK (also known as RIP2), a caspase-recruitment domain-containing kinase, plays a critical role in the NF-κB activation in response to Nod1 and Nod2 stimulation (Inohara et al., 2000; Park et al., 2007). RICK also undergoes K63-linked ubiquitination upon Nod1 and Nod2 stimulation at K209 located within its kinase domain. This ubiquitination triggers the oligomerization of RICK and the recruitment of TAK1 kinase, in turn facilitating IKK activation and secretion of cytokine and chemokine (Hasegawa et al., 2008). IRAK1, involved in TLR and IL-1R-mediated NF-κB activation, undergoes K63-linked ubiquitination upon IL-1 treatment, which is presumably ubiquitinated by E3 ligase TRAF6 (Conze et al., 2008). Ubiquitinated IRAK1 then recruits IKKγ and activates NF-κB signaling (Conze et al., 2008).

Stimulation of TNFR by the binding of TNFα triggers the recruitment of TRADD, TRAF2, and RIP1 to the receptor and also results in the activation of the canonical NF-κB pathway (Ermolaeva et al., 2008; Ha et al., 2009; Skaug et al., 2009; **Figure 2**). Similar to TRAF6, overexpressed TRAF2 is polyubiquitinated via K63-linked ubiquitination and is sufficient to induce NF-κB activation. However, there is functional redundancy between TRAF2 and TRAF5, as TRAF2-deficient mouse embryonic fibroblasts (MEFs) still activate NF-κB in response to TNFα, whereas TRAF2/5 compound mutant MEFs is compromised in IKK activation (Yeh et al., 1997; Grech et al., 2004; Skaug et al., 2009; Zhang et al., 2009). RIP1 is also polyubiquitinated via K63-linked chains by TRAF2/5 and cIAP1/2 complex, which is required for TNFα-induced NF-κB activation (Varfolomeev et al., 2008; Bertrand et al., 2011). The polyubiquitin chains on RIP1 serve as docking sites to recruit the TAK1 and IKK complexes to TNFR because the RIP1-K377R mutant failed to recruit TAK1 and IKK to the receptor, while retained the ability to recruit the adaptor protein TRADD or TRAF2 (Ea et al., 2006). However, the identification of free and unanchored K63-linked polyubiquitin chains directly activating TAK1 and IKK in the IL-1R/TLR pathway (Xia et al., 2009) suggests that such a mechanism may also exist in the TNFR pathway.

Interestingly, a recent report identified a novel mechanism by which enteropathogenic *Escherichia coli* NleE, a conserved bacterial type-III-secreted effector, regulates ubiquitin-chain signaling and its specificity in NF-κB activation during infection (Zhang et al., 2012). Specifically, NleE was found to possess *S*-adenosyl-L-methionine-dependent methyltransferase activity and modified a zinc-coordinating cysteine in the Npl4 zinc finger (NZF) domains in TAB2 and TAB3, which led to the loss of zinc ion and their ubiquitin-chain binding activity and subsequent inactivation of host NF-κB signaling activation.

On the other hand, the non-canonical NF-κB pathway regulation depends on tightly controlled degradation of NIK. TRAF3, TRAF2, and cIAP promote rapid proteasomal degradation of NIK by the ubiquitin–proteasome system (UPS). Therefore, under basal conditions, NIK protein is almost undetectable (Sasaki et al., 2008; Vallabhapurapu et al., 2008; Zarnegar et al., 2008a,b; Razani et al., 2010). However, various stimuli, such as CD40L and BAFF, trigger TRAF2-mediated K63-polyubiquitination of cIAP, which promotes K48-linked ubiquitination of TRAF3 and targets it to the proteasome (Vallabhapurapu et al., 2008; Zarnegar et al., 2008b). NIK is released from TRAF3 inhibition and phosphorylates and activates IKKα, which then induces phosphorylation of p100 and its subsequent processing by proteasome (Skaug et al., 2009; Sun, 2011). The processing of p100 is an important step in the activation of non-canonical NF-κB pathway, which not only generates p52 transcription factor, but also induces the nuclear translocation of RelB/p52 by releasing from its cytoplasmic localizing sequence present in the digested fragment (Skaug et al., 2009; Sun, 2011).

Interestingly, DDR can results in NF-κB activation via K63-linked ubiquitination as well as a novel linear ubiquitination. It has been shown that the E3 ligase linear ubiquitin-chain assembly complex (LUBAC), regulates NF-κB activation upon genotoxic stress by promoting linear ubiquitination of NEMO (Niu et al., 2011). In addition, ATM- and NEMO-dependent ELKS ubiquitination coordinates TAK1-mediated IKK activation in response to genotoxic stress (Wu et al., 2010). Furthermore, a cytoplasmic ATM–TRAF6–cIAP1 module links nuclear DNA damage signaling to ubiquitin-mediated NF-κB activation (Hinz et al., 2010).

Since the process of ubiquitination of target proteins is tightly regulated by the activities of DUBs, it is conceivable that DUBs also play critical role in the regulation of NF-κB signaling. CYLD, a tumor suppressor which is found to be mutated in patients with familial cylindromatosis in which affected individuals develop multiple benign tumors of the skin appendages (Bignell et al., 2000), shows specific DUB activity toward K63-linked polyubiquitin chains, negatively regulating IKK activation. CYLD has been shown to target TRAF6, TRAF2, and NEMO for deubiquitination (Brummelkamp et al., 2003; Kovalenko et al., 2003; Mauro et al., 2006; Sun, 2008, 2010; Bhoj and Chen, 2009), thus presumably reduced their activities in NF-κB activation. Furthermore, CYLD also cleaves free unanchored K63-linked polyubiquitin chains, thus inhibiting TAK1 and IKK activation (Xia et al., 2009). Importantly, the tumor suppressive function of CYLD has been demonstrated *in vivo* using genetically engineered mouse (GEMs) model. *Cyld*-deficient mice are highly susceptible to chemical-induced skin tumors, chemical-induced colitis, and colon-tumorigenesis (Massoumi et al., 2006; Reiley et al., 2006, 2007; Zhang et al., 2006).

Another DUB, A20, which contains an N-terminal OTU-type DUB domain and seven C-terminal zinc fingers, has been suggested to play a critical role in the negative regulation of NF-κB activation, and A20-deficient mice have hyperactivation of NF-κB pathway and multi-organ inflammation (Brummelkamp et al., 2003; Kovalenko et al., 2003; Mauro et al., 2006; Sun, 2008, 2010; Bhoj and Chen, 2009). Recent evidence also implicates that A20 also plays an essential role in human diseases (Hymowitz and Wertz, 2010). For example, polymorphisms in the A20 locus are associated with multiple autoimmune diseases including systemic lupus erythematosis. In addition, A20 acts as a tumor suppressor in B cell lymphoma (Compagno et al., 2009; Kato et al., 2009) and mutated in marginal zone lymphomas (MZLs) (Novak et al., 2009). A20 can deubiquitinate RIP1 via its OTU domain and also act as an E3 ligase to add K48 polyubiquitin chains to RIP1 via the ZnF region to promote its proteasomal degradation (Wertz et al., 2004). In addition, A20 has been shown to promote disassembly of ubiquitination complexes in the IL-1R and TNFR pathways, including TRAF6–UBC13, cIAP1/2–UBC13, and cIAP1/2–UBCH5, as well as proteasomal degradation of UBC13

and UBCH5 (Shembade et al., 2009, 2010). Furthermore, A20 has also been reported to block recruitment of the adaptor proteins TRADD and RIP1 to TNFR (He and Ting, 2002), and to promote lysosomal degradation of TRAF2 (Li et al., 2009). Interestingly, A20 is recently shown to inhibit IKK activation through direct and non-catalytic mechanism (**Figure 2**). Specifically, A20 can bind to the unanchored polyubiquitin chain via its ZnF7 domain and interacts with IKKγ/NEMO, which results in an impairment of TAK1-mediated phosphorylation of IKK. Importantly, the catalytic Cys 103 residue of A20 is not required for the inhibition of IKK activation (Bosanac et al., 2010; Skaug et al., 2011).

Recently, it has been demonstrated that E3 ligase ITCH and CYLD formed a complex which can sequentially cleave K63-linked ubiquitin-chain and catalyzes K48-linked ubiquitination to deactivate TAK1 and terminate NF-κB signaling (Ahmed et al., 2011), providing an example of how K48 and K63-linked ubiquitinations are closely linked and can be differentially utilized to control kinase activation and deactivation.

K63-UBIQUITINATION PLAYS AN IMPORTANT ROLE IN Akt KINASE ACTIVATION

Protein kinases play a pivotal role in signaling transduction cascade, and they act as signaling mediators to convey the extracellular clues, such as growth factors and cytokines, from the outside of the cells to the nucleus by inducing protein phosphorylation (Hynes and MacDonald, 2009; Klein and Levitzki, 2009). The level of phosphorylation of a target protein is counteracted by the activity of phosphatase. Protein kinases are also subjected to phosphorylation, which regulates their activities and expressions. Recently, emerging evidence suggests a critical role for K63-linked ubiquitination in the regulation of kinase activation (Yang et al., 2009, 2010a,b). Here we will focus on the regulatory function of K63-linked polyubiquitination in kinase activation using AKT as an example. AKT (also known as PKB) is a serine/threonine protein kinase, which is a keystone in growth factor and cytokine-mediated signaling cascade (Datta et al., 1999; Brazil et al., 2002; Cantley, 2002; Gonzalez and McGraw, 2009; Yang et al., 2010a). Giving that Akt regulate numerous biological functions, such as cell growth, survival, cell migration, and metabolism, by phosphorylating several downstream effectors, the activity of Akt must be stringently controlled. Aberrant Akt activation is observed in various human cancers, and importantly, Akt1, Akt2, and Akt3 isoforms are found to be overexpressed in human cancers (Staal, 1987; Cheng et al., 1992, 1996; Bellacosa et al., 1995; Nakatani et al., 1999; Stahl et al., 2004). Recent studies show that Akt1 mutations are observed in a subset of human cancers and are associated with Akt hyperactivation (Carpten et al., 2007; Kim et al., 2008a; Malanga et al., 2008; Mohamedali et al., 2008; Askham et al., 2010; Shoji et al., 2009; Zilberman et al., 2009). The role of Akt in cancer development has been supported by numerous animal tumor models. For example, $Pten^{+/-}$ mice with aberrant Akt activation develop multiple tumors, which can be inhibited by Akt1 deficiency (Di Cristofano et al., 2001; Chen et al., 2006). In addition, the prostate-specific expression of constitutively active Akt1 in mice leads to prostate intraepithelial neoplasia (Majumder et al., 2003, 2004). Accordingly, these results highlight the critical role of the PI3K/Akt pathway in cancer development.

Ubiquitination has been shown to regulate Akt protein degradation. Several E3 ligases, such as CHIP (carboxyl terminus of Hsc-70 interacting protein), BRCA1, and TTC3, have been shown to target AKT for ubiquitination and subsequent proteasome degradation (Dickey et al., 2008; Xiang et al., 2008; Suizu et al., 2009; Toker, 2009). In addition, mTORC2-mediated phosphorylation of Akt at T450 also regulates Akt stability as Sin1 deficiency or mTOR knockdown results in reduced T450 phosphorylation and increased Akt ubiquitination and degradation (Facchinetti et al., 2008).

Recently, TRAF6 has been identified as a unique E3 ligase for Akt and induces K63-linked Akt ubiquitination, in turn facilitating Akt membrane recruitment and subsequent Akt T308 phosphorylation and activation (Yang et al., 2009, 2010a). The notion is supported by the fact that primary MEFs deficient for Traf6 display profound defects in Akt membrane recruitment and activation in response to various stimuli such as growth factors and cytokines. The ubiquitination of Akt occurs on the K8 and K14 residues within the PH (pleckstrin homology) domain of Akt. Notably, Akt K8R and Akt K14R mutants display defects in Akt ubiquitination, correlated with the impairment of Akt membrane recruitment and T308 phosphorylation, suggesting that Akt ubiquitination plays an important role in Akt membrane recruitment and activation.

Analysis of K8R mutant of Akt reveals that the regulation of Akt membrane recruitment by Akt ubiquitination does not result from its impact on the PIP3 binding. It is known that Akt membrane localization is essentially dependent on its binding to phosphoinositol (3,4,5) trisphosphate (PIP3) in the plasma membrane (Brazil et al., 2002; Varnai et al., 2005). The K14R Akt mutant fails to bind to PIP3, as the K14 residue is within the PIP3 binding pocket. However, the K8 residue is not within the PIP3 binding pocket (Bellacosa et al., 1998; Rong et al., 2001; Carpten et al., 2007) and there is no difference in the binding to PIP3 between the Akt K8R mutant and the wild-type (Wt) Akt (Yang et al., 2009).

One Akt mutant (E17K), found in a subset of human cancers, displays higher PIP3 binding, membrane localization, and activation (Brazil et al., 2002; Varnai et al., 2005). Interestingly, this cancer-associated Akt mutant also displays a hyperubiquitination of Akt, and the blocking of this hyperubiquitination profoundly impairs Akt membrane localization, phosphorylation, and activation (Carpten et al., 2007; Yang et al., 2009). These results suggest that mutation of E17 residue to lysine residue in Akt results in not only hyperubiquitination but also hyper-PIP3 binding, and both of these events appear to contribute to the constitutive membrane localization and activation of Akt. Collectively, these results demonstrate that TRAF6-mediated Akt ubiquitination is relevant and important for human cancers. Along this line, another Akt mutation E49K has been recently identified in bladder cancer, which also displays Akt hyperphosphorylation and activation compared to WtAkt (Askham et al., 2010). Given this Akt mutation is located in the PH domain of Akt but not in the PIP3 binding pocket, we speculate that this mutation may not affect Akt binding to the PIP3. Since this Akt mutant gains an additional lysine residue, it is very likely that such mutation may also results in hyperubiquitination of Akt, in turn contributing to its hyperactivation and hyper-oncogenic potential.

Currently, it remains unclear how the K63-linked ubiquitination of Akt results in its recruitment to plasma membrane and subsequent activation. One possibility is that TRAF6-mediated polyubiquitination of Akt may increase its binding to PIP3 on the membrane (**Figure 3**). However, it may be not the case, since the Akt K8R mutant, which shows an impairment in Akt ubiquitination, membrane recruitment and activation, displays similar binding affinity to PIP3 as WtAkt does (Yang et al., 2009), suggesting that other mechanisms contribute to ubiquitination-mediated Akt membrane recruitment and activation. It has been shown that TCL1 family proteins form heterodimer with Akt and promote Akt dimerization, in turn augmenting Akt activation (Teitell, 2005). Therefore, another possibility is that K63-polyubiquitination of Akt may enhance the formation of TCL1–Akt complex and Akt dimerization, thus promoting Akt membrane localization and Akt activation (**Figure 3**). Furthermore, since K63-linked polyubiquitin chains have been proposed to serve as docking sites for many signaling molecules, it is highly possible that K63-ubiquitination of Akt enhances its binding to membrane bound adaptor protein containing ubiquitin-binding property, thereby facilitating Akt membrane localization and activation (**Figure 3**). More works will be required to further dissect these possibilities.

TARGETING UBIQUITINATION/DEUBIQUITINATION PATHWAYS FOR THERAPY

Ubiquitination and deubiquitination mediated through the E3 ligases and DUBs regulate the activities and functions of a large amount of protein, including tumor suppressors and oncogenes important for cell proliferation, apoptosis, and tumorigenesis. Importantly, the important roles of these proteins in tumorigenesis are supported by their frequent deregulations in human cancer and by mouse tumor models to reveal their important roles in cancer development. Therefore, targeting these proteins may have important therapeutic implications for the treatment of human cancer. Efforts have been invested to target the proteasome subunits (the proteolytic 20S subunit and the regulatory 19S subunit), and E3 ubiquitin ligase, and DUBs.

Bortezomib, an inhibitor proteasome 20S subunit, has been approved by the FDA for the treatment of multiple myeloma (Adams and Kauffman, 2004; Bold, 2004). Recently, the small molecule b-AP15, which inhibits the activity of two 19S regulatory-particle-associated DUBs, ubiquitin C-terminal hydrolase 5 (UCHL5), and ubiquitin-specific peptidase 14 (USP14), can induce tumor cell apoptosis that is insensitive to TP53 status and overexpression of the apoptosis inhibitor BCL2. Furthermore, b-AP15 inhibits tumor progression in four different *in vivo* solid tumor xenograft models, including FaDu squamous carcinoma, HCT-116 colon carcinoma, Lewis lung carcinomas (LLCs), orthotopic breast carcinoma (4T1), and also inhibits organ infiltration in one acute myeloid leukemia model (C1498 leukemia; D'Arcy et al., 2011).

Many E3 ligases have been shown to play an oncogenic role in tumorigenesis, therefore, E3 ubiquitin ligases, which confer substrate specificity, are attractive targets for human cancers. For example, efforts have been invested on targeting HDM2, the E3 ligase for tumor suppressor p53, aiming to elevate the protein level of p53. Nutlin 3/R7112, an inhibitor that disrupts the interaction between HDM2 and its substrate p53, has entered clinical trials

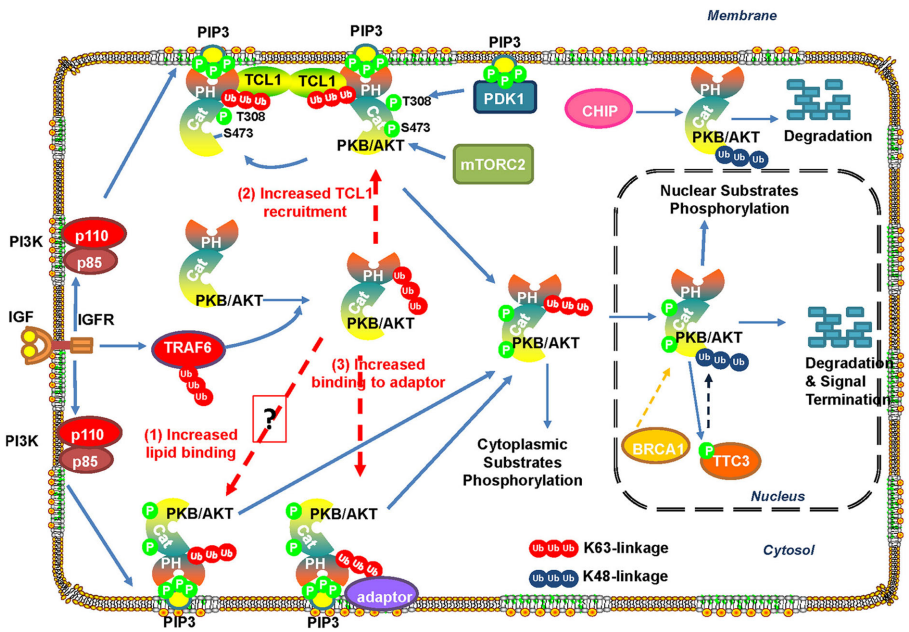

FIGURE 3 | Ubiquitination regulates Akt stability and activation. IGF-1-induced activation of IGF-1R promotes TRAF6 activation, which then triggers K63-linked ubiquitination of Akt and promotes Akt membrane recruitment and subsequent phosphorylation by PDK1 and mTORC2. Active Akt then phosphorylates its substrates in the cytoplasm and nucleus to exert its biological functions. E3 ligase TTC3 has been suggested to play a role in the termination of active Akt signaling in the nucleus by promoting Akt ubiquitination and degradation by the proteasome. TTC3 activity is regulated by Akt-mediated phosphorylation at S378, thus providing a negative feedback loop for Akt inactivation. In addition, Akt ubiquitination is promoted by CHIP or BRCA1 and leads to Akt degradation.

(Cohen and Tcherpakov, 2010). In addition, several inhibitors that target inhibitors of apoptosis proteins (IAPs) have also entered clinical trials (Cohen and Tcherpakov, 2010). These inhibitors induce the autoubiquitination and degradation of the IAPs and promote cancer death by stimulating the TNF-α pathway (Wu et al., 2007).

Given the critical role of TRAF6 in the regulation of Akt ubiquitination, membrane localization, and activation (Yang et al., 2009), small molecules targeting TRAF6 can suppress Akt signaling and may be considered as potential or adjuvant agents for cancer therapy. In line with this notion, TRAF6 inhibition by shRNA knockdown reduces Akt activation in prostate cancer cells, suppresses tumor formation in the xenograft tumor model, and potentiates apoptosis induced by chemotherapy agents (Yang et al., 2009). Therefore, TRAF6 can be considered as a potential therapeutic target for human cancer. In addition to small molecules inhibitors for TRAF6, miR-145, and miR-146a have shown to repress TRAF6 protein translation and display a potent tumor suppressive effect on restricting the formation of primary and metastatic breast cancer (Hou et al., 2009; Hurst et al., 2009; Starczynowski et al., 2010; Yuan et al., 2010). Therefore, miR-145 and miR-146a can be applied as another strategy to target TRAF6 for cancer treatment.

Deubiquitinating enzymes are another class of potential drug targets for cancer treatment. DUBs have been shown to play an important role in the regulation of the stability of many oncoproteins by counteracting the activity of E3 ligase. USP7 (ubiquitin-specific protease 7), which deubiquitinates HDM2, can lead to increased levels of HDM2 and decreased levels of p53. Therefore, inhibitors for USP7 will promote HDM2 degradation and p53 accumulation. Interestingly, as USP7 silencing stabilizes p53 and enhances its activity, small molecular inhibitors for USP7 are expected to suppress cancer cell growth. As expected, a novel USP7 inhibitor indeed induces p53-dependent apoptosis by stabilizing p53 (Colland et al., 2009). USP20, also called VDU2 (von Hippel–Lindau deubiquitinating enzyme 2) stabilizes hypoxia-inducible factor 1α (HIF-1α; Li et al., 2005), which is overexpressed in many human cancers. Therefore, targeting USP20 can induce degradation of HIF-1α and be a potential drug target for human cancer. Furthermore, many transcription factors have been demonstrated to be overexpressed and play potent oncogenic functions in tumorigenesis, such as Myc (Pelengaris et al., 2002), CyclinD1 (Musgrove et al., 2011) and inhibitors of DNA binding (ID; Hasskarl and Munger, 2002). However, it has been well-accepted that it is difficult to target transcription factors with small molecules since they lack enzymatic activity, although there is a report that small molecule has been designed to target Bcl-6 in mouse model for cancer therapy (Cerchietti et al., 2010). Targeting Myc is recently demonstrated to be very effective in the treatment of human cancers (Adhikary and Eilers, 2005; Soucek et al., 2008; Herold et al., 2009). In addition, inactivation of Cyclin D1 can abolish breast cancer development driven by oncogenic Ras or Neu (Lee et al., 2000; Yu et al., 2001). Further, IDs overexpression is observed in a broad range of dedifferentiated primary human malignancies, such as pancreatic carcinoma and neuroblastoma (Perk et al., 2005). Since many of these oncogenic transcription factors are short-lived oncoproteins undergoing fast turnover, therapeutic strategies by targeting their corresponding DUBs will be an alternative approach, which will lead to rapid degradation of these oncoproteins. USP28, USP2, USP1, Dub3, and USP9x have been shown to play an important role in stabilizing oncogenic signals, such as Myc (Popov et al., 2007), Cyclin D1 (Shan et al., 2009), and IDs (Williams et al., 2011) and CDC25A (Pereg et al., 2010), MCL-1 (Opferman and Green, 2010; Schwickart et al., 2010) respectively. Therefore, small molecules inhibitors to target these DUBs are theoretically possible to design and to target these oncogenic transcription factors indirectly. Future experiments using small molecule inhibitors to target these DUBs in preclinical mouse tumor models may provide *in vivo* evidence that targeting the DUBs can be ideal therapeutic strategies for the treatment of human cancers.

CONCLUDING REMARKS

A large amount of recent studies in the area of ubiquitination study have revealed several novel roles of ubiquitination in signaling transduction pathways involved in a broad range of biological functions, such as DDR, NF-κB activation, and kinase activation. Ubiquitination can play a distinct role in the regulation of protein fate, depending on which type of the polyubiquitin chains formed within the proteins (**Figure 1**). Mono-ubiquitination plays a role in endocytosis, protein transport, DNA repair, and histone regulation. K48-linked polyubiquitination signals mainly target proteins for proteasome-dependent protein degradation, whereas K63-linked ubiquitination serves as a scaffold for protein/protein interaction, in turn regulating the localization and activity of protein kinase. In addition, a novel type of linear polyubiquitination plays a role in the NF-κB activation. It is anticipated that other novel functions on this posttranslational modification will be uncovered in the near future.

As strong evidence has demonstrated an important role for ubiquitination in cancers, efforts to specifically target this pathway with small molecules inhibitors have now been actively investigated. Proteasome inhibitor Bortezomib has been approved to be used in the treatment of multiple myeloma. Several small molecule inhibitors targeting E3 ligases have entered clinical trials, such as inhibitors for HDM2 and IAPs. In addition, efforts have been invested to target DUBs and small molecule inhibitors have been developed and demonstrated efficacy in the treatment of human cancer. We anticipate to see more and more small molecule inhibitors targeting this pathway will be developed and eventually utilized for the treatment of human cancers.

However, there still remains several outstanding questions and warrant for future investigations. Currently only a few E3 ligases are able to trigger K63-linked ubiquitination. Will more such E3 ligases be discovered in the genome? As many proteins can undergo both K48- and K63-linked ubiquitination, it remains to be determined how the E3 ligases and DUBs coordinate to regulate signaling activation for protein kinases. A recent report on ITVH–CYLD sheds light on how K48- and K63-link ubiquitination/deubiquitination are closely coupled to activate and inactivate TAK1 to tightly control the activation of NF-κB signaling pathway. In addition, it remains largely unknown for the roles of the novel linear polyubiquitin chains in the regulation of target protein function. Moreover, much work is needed

to determine the mechanisms for the regulation of linear vs. branch polyubiquitination and how the ubiquitination regulates the localization and activation of protein kinase. Furthermore, a deeper mechanistic understanding of ubiquitination and deubiquitination and kinase regulation by ubiquitin remains as an important challenge for this field. In addition, we need to understand how substrate binding and activity of ubiquitination and DUBs are regulated. Addressing all these important questions will lead to comprehensive understanding of the complicated mode of ubiquitination and deubiquitination in regulating signaling transduction pathways.

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Skp2 is a promising therapeutic target in breast cancer

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Breast cancer is the most common type of cancer among American women, and remains the second leading cause of cancer-related death for female in the United States. It has been known that several signaling pathways and various factors play critical roles in the development and progression of breast cancer, such as estrogen receptor, Notch, PTEN, human epidermal growth factor receptor 2, PI3K/Akt, BRCA1, and BRCA2. Emerging evidence has shown that the F-box protein S-phase kinase associated protein 2 (Skp2) also plays an important role in the pathogenesis of breast cancer. Therefore, in this brief review, we summarize the novel functions of Skp2 in the pathogenesis of breast cancer. Moreover, we provide further evidence regarding the state of our knowledge toward the development of novel Skp2 inhibitors especially natural "chemopreventive agents" as targeted approach for the prevention and/or treatment of breast cancer.

Keywords: Skp2, breast cancer, therapy, signaling pathway, ubiquitination, cell cycle

INTRODUCTION

Breast cancer is the most common female malignancy, and the second leading cause of cancer-related death after lung cancer in the United States (Siegel et al., 2011). The American Cancer Society estimates that approximately 230,480 American women will be diagnosed with breast cancer and almost 40,000 will die from it in 2011 (Siegel et al., 2011). Breast cancer has been clinically characterized by the expression of hormone and growth factor receptors such as estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (Her2; Lin et al., 2010). Approximately 70% of breast cancer patients have elevated expression of ER and PR, while 20% of breast tumors have overexpression of Her2. The remaining 10% of breast cancer cases are classified as triple-negative breast cancers due to the lack of expression of ER, PR, and Her2 (Lin et al., 2010). Even though breast cancer recurrence rates have been significantly decreased due to early detection and adjuvant therapies, breast cancer still causes high mortality. Current therapies for breast cancer include surgery, radiation, chemotherapy, hormonal, and biological therapies (Lin et al., 2010). Despite improved treatments that have been achieved recently, many breast tumors are not eradicated effectively due to their intrinsic or acquired resistance, or relapse following initial response, resulting in metastatic disease at later stages that leads to patient death (Lin et al., 2010). Therefore, this disappointing outcome suggests that further understanding of the molecular mechanisms underlying the development of breast cancer, especially the critical events of the metastatic spread, is essential to identify new therapeutic targets for achieving better treatment of breast cancer.

In recent years, it has been demonstrated that multiple cellular signaling pathways including the ER, PR, epidermal growth factor receptor (EGFR), breast cancer susceptibility gene (BRCA), PI3K (phosphatidylinositol 3-kinase), PTEN (phosphatase and tensin homolog on chromosome 10), mammalian target of rapamycin (mTOR), MAPK (mitogen-activated protein kinases), androgen receptor (AR), Akt, nuclear factor-κB (NF-κB), receptor of activated nuclear factor kappa ligand (RANKL), Wnt, sonic hedgehog (SHH), platelet-derived growth factor (PDGF), and Notch pathways have been found to play important roles in the development and progression of breast cancer (Foley et al., 2010; Haagenson and Wu, 2010; Nahta and O'Regan, 2010; Narod, 2010; Prosperi and Goss, 2010; Rosen et al., 2010; Visbal and Lewis, 2010; Guo et al., 2011; Hernandez-Aya and Gonzalez-Angulo, 2011; O'Regan and Hawk, 2011). It is worth noting that exact mechanisms by which breast cancer arises remain largely unclear. Emerging evidence has demonstrated that S-phase kinase associated protein 2 (Skp2) is critically involved in the pathogenesis of breast cancer (Hulit et al., 2006; Sun et al., 2007; Fujita et al., 2008; Voduc et al., 2008; Chan et al., 2010b). Therefore, in this review article, we will provide an overview on the role of Skp2 oncoprotein, and summarizing recently published literatures that highlight the novel roles of Skp2 in mammary tumorigenesis. Finally, we will also summarize approaches to inhibit Skp2 and would suggest that Skp2 could be a promising therapeutic target in combating human breast cancer.

Skp2 IS ONE OF THE COMPONENTS OF THE SCF E3 LIGASE COMPLEX

S-phase kinase associated protein 2 belongs to ubiquitin–proteasome system (UPS) that plays vital roles in regulating many biological processes by controlling the timely turn-over of proteins (Frescas and Pagano, 2008). UPS includes three types of enzymes for exerting UPS function via a cascade of enzymatic reactions (Nalepa et al., 2006). The three enzymes are ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (UBC, also known as E2), and ubiquitin ligase (E3; Weissman et al., 2011). The initial step is to add ubiquitin to E1 and subsequently transfer of it to the E2 enzyme. The ubiquitin-charged E2 then interacts with E3 and

transfers the ubiquitin onto the substrate, leading to the formation of a mono-ubiquitin or polyubiquitin chain due to the consecutive addition of ubiquitin moieties to target proteins (Nakayama and Nakayama, 2006). It is evident that the specificity of substrate selection depends on the E3, leading to substrate degradation by the 26S proteasome (Nakayama and Nakayama, 2006). This biochemical feature could explain why deregulation of E3 has been frequently found in carcinogenesis, whereas the function of E1 and E2 in carcinogenesis documented only few published reports (Okamoto et al., 2003).

It is known that Skp2 is one of the components of the Skp1–Cullin1–F-box (SCF) E3 ligase complex. The SCF complexes comprise a family of multi-subunit E3 ubiquitin ligases that target selected proteins for destruction by the 26S proteasome degradation (Frescas and Pagano, 2008). SCF complexes contain several constant core proteins such as Skp1, Rbx1 (also known as ROC1), Cullin1, and a variable subunit called F-box protein (Frescas and Pagano, 2008). F-box protein that functions as a receptor for target proteins can bind to a specific subset of substrates for promoting their degradation (Nakayama and Nakayama, 2006). So far, more than 70 putative F-box proteins encoded in the human genome have been found (Nakayama and Nakayama, 2006). It is clear that Skp2 is an F-box protein of the SCF complex (**Figure 1**). Because Skp2 is originally discovered with Skp1 associated with the S-phase kinase Cdk2/cyclin A, it was named as Skp2 (Frescas and Pagano, 2008). Among the many F-box proteins, Skp2 has been well-characterized and it has been shown to be involved in carcinogenesis.

Skp2 IS A PROTO-ONCOPROTEIN

S-phase kinase associated protein 2 is the specific factor of the SCF^{skp2} E3 ligase involved in cell cycle progression through degradation of its targets (Bashir and Pagano, 2004; Frescas and Pagano, 2008). For example, Skp2 is essential for p27 degradation and thereby limiting cells in G1 phase, prior to entry into S-phase (Amati and Vlach, 1999; Carrano et al., 1999; Tsvetkov et al., 1999). This finding raised the question of whether the observed lower levels of p27 are caused by overexpression of Skp2 in human cancers. Indeed, accumulating evidence has shown that Skp2 expression is inversely correlated with p27 levels in different types of human malignancies (Hershko and Shapira, 2006). To date, in addition to p27, specific substrates of Skp2 have also been identified which include p57 (Kamura et al., 2003), p21 (Yu et al., 1998), p130 (Tedesco et al., 2002; Bhattacharya et al., 2003), Tob1 (Hiramatsu et al., 2006), FOXO1 (Huang et al., 2005), and many others (Michel and Xiong, 1998; Yu et al., 1998; Nakayama et al., 2000). These substrates are involved in many cellular processes such as cell cycle regulation, proliferation, differentiation, apoptosis, and survival. Without a doubt, Skp2 has important functions in the regulation of these cellular processes due to degradation of its substrates, most of which are tumor suppressor proteins (Gstaiger et al., 2001). Because Skp2 is responsible for the degradation of the above-mentioned tumor suppressor proteins, Skp2 is thought to function as oncoprotein (Gstaiger et al., 2001).

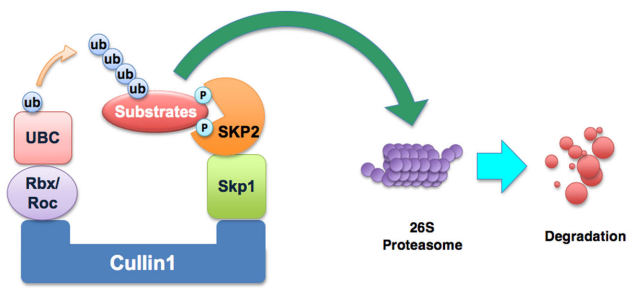

FIGURE 1 | Illustration of the SCF^{skp2} complex. The SCF (Skp1–Cullin1–F-box) E3 ubiquitin ligase complex consists of four components: Skp1, Rbx1, Cullin1, and the variable F-box protein. In SCF^{skp2}, the F-box protein component is Skp2. Skp2 recognizes the targeted proteins and makes the ubiquitin transfer to the substrate protein by UBC (the E2 enzyme). The addition of polyubiquitin targets substrates for degradation by the 26S proteasome. The known substrates of Skp2 include p21, p27, p57, p130, Tob1, FOXO1, and c-Myc.

Many studies have shown that overexpression of Skp2 is observed in a variety of human cancers, including lymphomas (Seki et al., 2003), prostate cancer (Wang et al., 2011a), colorectal cancer (Li et al., 2004), melanoma (Rose et al., 2011), nasopharyngeal carcinoma (Fang et al., 2009; Xu et al., 2011), pancreatic cancer (Schuler et al., 2011), and breast carcinomas (Radke et al., 2005; Zheng et al., 2005). Moreover, Skp2 has been found to be a prognostic marker in multiple cancers. For example, overexpression of Skp2 is associated with late metastases to lymph nodes leading to poor survival in colorectal cancer (Li et al., 2004). A separate study showed that overexpression of Skp2 was associated with significantly poorer tumor differentiation and reduced patient survival in gastric cancer (Ma et al., 2005). Similarly, Skp2 expression was correlated with histological grade and tumor size in hepatocellular carcinoma (Lu et al., 2009). Moreover, elevated Skp2 expression is related with tumor metastasis in melanoma (Rose et al., 2011), lymphoma, oral squamous cell carcinomas (Tosco et al., 2011), pancreatic cancer (Einama et al., 2006), and prostate cancer (Wang et al., 2011a). Furthermore, Skp2 expression has been considered as a biomarker for poor prognosis in breast cancer (Voduc et al., 2008), melanoma (Rose et al., 2011), and nasopharyngeal carcinoma (Xu et al., 2011). Interestingly, one recent report showed that Skp2 mediates resistance of pancreatic cancer cell lines toward the TRAIL (tumor factor-related apoptosis-inducing ligand)-induced apoptosis (Schuler et al., 2011). Taken together, Skp2 is believed to be a proto-oncoprotein. In the following paragraphs, we will discuss the role of Skp2 in the breast cancer progression.

THE ROLE OF Skp2 IN DEVELOPMENT AND PROGRESSION OF BREAST CANCER

A growing body of literature strongly suggests that Skp2 plays critical roles in the breast tumorigenesis. For example, Skp2 has been reported to be overexpressed in a subset of breast carcinomas with low level of p27 expression (Signoretti et al., 2002). Emerging evidence also reveals that Skp2 plays important roles in cell growth, apoptotic cell death, invasion, and metastasis in human breast cancer (Zheng et al., 2005; Sonoda et al., 2006). Multiple signaling pathways, such as phosphatidylinositol 3-kinase (PI3K)/Akt (Gao et al., 2009a), ERK (extracellular signal-regulated kinase; Lin and Yang, 2006), peroxisome proliferator-activated receptor-γ

(PPARγ; Zaytseva et al., 2008), IGF-1 (insulin-like growth factor-1; Lu et al., 2004), and mTOR (Shapira et al., 2006) signaling have been discovered to cross-talk with Skp2, and thus it is believed that the cross-talks between Skp2 and these signaling pathways may play important roles in breast cancer. However, the molecular mechanism(s) by which Skp2 facilitates breast cancer remain largely elusive. Here, we will discuss some recent advances in the understanding on the role of Skp2 in breast tumor progression. Therefore, in the following sections, we will summarize the results of emerging studies on the Skp2 as well as its therapeutic implication in human breast cancer.

Skp2 IS OVEREXPRESSED IN BREAST CANCER

It has been widely accepted that Skp2 is frequently overexpressed in a variety of human cancers including breast cancer. For instance, both Skp2 mRNA and protein display elevated levels in breast cancer cell lines and primary breast tumors (Radke et al., 2005; Fujita et al., 2008). Specifically, using microarray analysis and immunohistochemistry, Pagano's group found that higher levels of Skp2 are present more frequently in ER-negative tumors than in the ER-positive cases (Signoretti et al., 2002). Moreover, Skp2B, an isoform of Skp2, is also overexpressed in breast cancer cell lines and primary breast tumors (Radke et al., 2005). Since Skp2 is overexpressed in human breast cancer, inhibition of Skp2 could be a promising therapeutic strategy for breast cancer treatment.

Skp2 PROMOTES CELL GROWTH IN BREAST CANCER

Emerging evidence is accumulating showing that Skp2 promotes cancer cell growth including breast cancer cell growth. For example, down-regulation of Skp2 by RNA interference significantly inhibited cell proliferation in MCF-7 breast cancer cells (Sun et al., 2007). Treatment with Skp2 siRNA followed by treatment with epirubicin further inhibited the proliferation of breast cancer cell lines (Sun et al., 2007). Consistent with the role of Skp2 in cell growth, Signoretti et al. (2002) found that inhibition of Skp2 induced a decrease of adhesion-independent growth in both ER-positive and ER-negative breast cancer cells. A study from Wan's group has also shown that overexpression of Skp2 enhanced cell proliferation in normal breast cell line MCF10A, while depletion of Skp2 reduces cellular growth in breast cancer cell line (Fujita et al., 2008). Lee and McCormick (2005) also found similar results, which showed that down-regulation of Skp2 inhibits the *in vitro* growth of glioblastoma cells. Moreover, knockdown of endogenous Skp2 by siRNA treatment also inhibited the *in vivo* tumor growth in nude mice (Sun et al., 2007). Furthermore, xenograft expressing high levels of Skp2B grows faster than xenograft expressing low levels of Skp2B (Radke et al., 2005), suggesting that Skp2B could also promote breast tumor growth. Recently, it has been found that Skp2B interacts with the REA (repressor of estrogen receptor activity) and that overexpression of Skp2B leads to a decreased REA levels, suggesting that Skp2B contributes to breast cancer in part by modulating the activity of the ER (Umanskaya et al., 2007). More recently, Chander et al. (2010) demonstrated that Skp2B attenuates the p53 activity by degradation of prohibitin, suggesting that his effect is independent of p300 in breast cancer.

Skp2 INHIBITS CELL APOPTOSIS IN BREAST CANCER

Inhibition of cell growth by down-regulation of Skp2 raised one question of whether the observed cell growth inhibition is caused by a possible increase in cellular apoptosis. Multiple studies have addressed this question. Indeed, Skp2 has been found to be involved in regulating cellular apoptosis in various types of human cancer cells (Kitagawa et al., 2008). Kitagawa et al. (2008) demonstrated that reducing the expression of Skp2 increased DNA-damage-mediated apoptosis in multiple cancer cells, while overexpression of Skp2 suppressed p53-mediated apoptosis. The reason is that Skp2 suppressed p300-mediated acetylation of p53 and subsequent transactivation ability of p53 through forming a complex with p300 (Kitagawa et al., 2008). It has also been reported that down-regulation of Skp2 caused apoptosis via induction of p27 in glioblastoma cells (Lee and McCormick, 2005). Moreover, down-regulation of both Skp2 and p27 increased apoptosis synergistically (Lee and McCormick, 2005). Huang et al. (2005) also found that overexpression of Skp2 inhibits transactivation of FOXO1 and abolishes the induced effect of FOXO1 on cell apoptosis in prostate cancer. Similar trends were found in breast cancer cell lines. For example, in breast cancer MCF-7 cells, knockdown of Skp2 by RNAi increased cellular apoptosis (Sun et al., 2007). Taken together, these results indicate that Skp2 could inhibit cell apoptosis in breast cancer cells. However, more thorough studies are required to fully understand the underlying molecular and signaling events by which Skp2 influences the cellular apoptotic decision.

Skp2 REGULATES CELL CYCLE IN BREAST CANCER

The cell cycle is tightly controlled by multiple regulatory mechanisms to ensure ordered and coordinated cell cycle progression. It is known that a major mechanism to ensure the orchestrated cell cycle is to degrade key regulators governing cell cycle progression by the UPS. SCF has been considered to be a major driving force controlling proper cell cycle progression through ubiquitination of G1 cyclins and CDK inhibitors (Skaar and Pagano, 2009). For example, Skp2 targets numerous substrates for degradation, many of which are negative cell cycle regulators such as p27 (Carrano et al., 1999; Tsvetkov et al., 1999), p57 (Kamura et al., 2003), p21 (Yu et al., 1998), p130 (Tedesco et al., 2002; Bhattacharya et al., 2003), and FOXO1 (Huang et al., 2005). Therefore, Skp2 has been found to correlate with dysregulation of cell cycle in human cancers including breast cancer. We have discovered that Akt regulates cell cycle through modulating Skp2 activity and its destruction by APC/Cdh1 (Gao et al., 2009a,b). Fujita et al. (2008) found that overexpression of Skp2 in MCF10A breast epithelial cells significantly elevated the fraction of cells in S-phase, suggesting that an increase in Skp2 protein levels could lead to an aberrant cell cycle. Consistent with these findings, the fraction of cells in S-phase was significantly reduced in Skp2-depleted MCF-7 breast cancer cells (Fujita et al., 2008). Moreover, Skp2 expression abrogates antiestrogen-mediated cell cycle arrest in hormone-dependent breast epithelial cancer cells (Signoretti et al., 2002). Further research toward exploration of the molecular mechanisms by which Skp2 regulates cell cycle requires in-depth investigations.

Skp2 PROMOTES TUMOR METASTASIS IN BREAST TUMOR

S-phase kinase associated protein 2 overexpression has been correlated with tumor progression such as stage and recurrence in human cancers (Einama et al., 2006), indicating that Skp2 may be important in cancer cell migration, invasion, and metastasis. Many studies have shown the positive relationship between Skp2 expression and tumor metastasis in human cancers (Tosco et al., 2011). For example, Tosco et al. (2011) found the correlation between Skp2 expression and nodal metastasis in oral squamous cell carcinomas. Hung et al. (2010) reported that Skp2 overexpression increased the expression of MMP-2 and MMP-9, leading to cell invasion in lung cancer cells. Consistent with this notion, Einama et al. (2006) also found that higher level Skp2 expression was correlated with the extent of lymph node metastasis in pancreatic ductal adenocarcinoma. Moreover, overexpression of Skp2 was detected more frequently in tumors metastatic to the axillary lymph nodes in breast cancer (Zheng et al., 2005), indicating that Skp2 could promote breast tumor metastasis. Recently, Lin's group identified one mechanism by which Skp2 increases tumor metastasis. Their elegant work demonstrated that Skp2 cooperates with Myc to induce RhoA transcription via recruiting Miz1 and p300 to the RhoA promoter (Chan et al., 2010a). Since RhoA plays a crucial role in cancer metastasis, deficiency of Skp2–Myc–Miz1–p300 transcriptional complex led to impaired RhoA expression, resulting in the inhibition of cell migration, invasion, and subsequent breast cancer metastasis (Chan et al., 2010a). Moreover, deficiency of Skp2 profoundly restricted breast cancer metastasis to the lung, whereas overexpression of Skp2 promoted the metastatic events (Chan et al., 2010a), suggesting a critical role for Skp2 in promoting breast cancer metastasis.

Skp2 PREDICTS FOR POOR PROGNOSIS IN BREAST CANCER

It has been reported that there is a statistically significant association between Skp2 expression levels and breast tumor grades. Moreover, high expression levels of Skp2 are associated with poor survival (Signoretti et al., 2002). Furthermore, Voduc et al. (2008) found that the combination of Skp2 and high cyclin E expression predicts poor prognosis in breast cancer and it is associated with high risk features. Davidovich et al. (2008) also demonstrated that Skp2 expression was inversely related to p27 levels, tumor grade, as well as expression of ER and PR. Both Skp2 and p27 were suggested to be accurate prognostic markers for disease-free and overall survival in breast cancer. Interestingly, one study showed that low p27 level and high Skp2 level were not associated with disease-free survival in breast cancer, although low p27 and high Skp2 were related to unfavorable prognostic factors including larger tumor size, higher grade tumor, ER- and PR-negative, and Her2 overexpression (Ravaioli et al., 2008). Taken together, it is obvious that further in-depth investigations are needed to confirm whether Skp2 could be a promising prognostic marker for breast cancer patients or not.

Skp2 IS INVOLVED IN DRUG RESISTANCE IN BREAST CANCER

Chemotherapy is the most important treatment strategy for human cancers including breast cancer. However, chemotherapy often fails to cure cancer due to the acquisition of drug resistance phenotype of cancer cells (Wang et al., 2010b). Thus, increasing drug sensitivity could offer better treatment for human cancer patients (Wang et al., 2011b). Recently, several studies have shown that Skp2 is involved in drug resistance in human cancers including breast cancer (Ishii et al., 2004; Davidovich et al., 2008; Schuler et al., 2011). A study from Schneider's group showed that Skp2 mediates resistance of pancreatic cancer cells toward TRAIL (tumor necrosis factor-related apoptosis-inducing ligand)-induced apoptosis (Schuler et al., 2011). Ishii et al. (2004) reported that overexpression of Skp2 increased chemoresistance against camptothecin, cisplatin, and AG1478 in human lung cancer cells. In support of this notion, similar trend was also found in breast cancer. Davidovich et al. (2008) demonstrated that high preoperative expression of Skp2 was correlated with resistance to cyclophosphamide/doxorubicin/5-fluorouracil therapy in 94% of breast cancer patients. Interestingly, overexpression of Skp2 is not associated with resistance to docetaxel in breast cancer patients (Davidovich et al., 2008), suggesting that Skp2 expression may be a useful marker for predicting response to doxorubicin-based preoperative chemotherapy (Davidovich et al., 2008). However, Ravaioli et al. (2008) found that the relative effects of chemo-endocrine versus endocrine therapy were similar regardless of the Skp2 expression status. Therefore, in order to better understand the precise role of Skp2 and its interrelationship with drug resistance, further in-depth investigations are required.

Skp2 CROSS-TALKS WITH OTHER MAJOR SIGNALING PATHWAYS IN BREAST CANCER

Although the molecular mechanism(s) by which Skp2 induces tumor growth has not been fully elucidated, multiple oncogenic pathways, such as mTOR, ERK, IGF-1, PPARγ, PI3K/Akt, and FoxP3 have been reported to cross-talk with Skp2 (Lu et al., 2004; Lin and Yang, 2006; Shapira et al., 2006; Zaytseva et al., 2008; Gao et al., 2009a). Thus, cross-talks between Skp2 and other pathways could play critical roles in mammary tumorigenesis. Interestingly, recent findings suggest that the tumor suppressor function of BRCA1 depends on its BRCT (BRCA C terminus) phosphoprotein binding motif, but not its E3 ligase activity (Shakya et al., 2011). Recently it was reported that Skp2 could modulate the activity of p53 in an E3 ligase-independent manner (Kitagawa et al., 2008), indicating that Skp2 could exert its oncogenic function in both an E3 ligase-dependent and independent manner. In the following paragraphs, we will discuss some recent advances on the role of Skp2 in tumor progression, especially its cross-talk with other signaling pathways.

THE ROLE OF mTOR PATHWAY IN THE REGULATION OF Skp2

The cross-talk between mTOR pathway and Skp2 pathway has been reported recently (Pene et al., 2002; Shapira et al., 2006). It is known that mTOR plays an important role in the regulation of cellular homeostasis, cell growth, and survival pathways (Bjornsti and Houghton, 2004). In order to fulfill the regulatory function, mTOR kinase assembles into two distinct complexes (mTORC1 and mTORC2). The mTORC1 consists of mTOR, Raptor, proline-rich Akt substrate of 40 kDa (PRAS40), and G-protein β-subunit-like protein (GβL). The well-characterized mTORC1 kinase substrates include S6K (p70 S6 ribosomal kinase) and phosphorylated 4E-binding protein 1 (4E-BP1; Bjornsti and Houghton,

2004). The mTORC2 is composed of mTOR, Rictor, GβL, protein observed with Rictor-1 (PROTOR), and stress-activated protein kinase interacting protein 1 (Sin1; Bjornsti and Houghton, 2004). Interestingly, the Raptor-containing complex is sensitive to rapamycin and regulates cell proliferation through phosphorylating S6K and 4E-BP1, whereas the Rictor-containing complex is not sensitive to rapamycin (Hay and Sonenberg, 2004; Inoki et al., 2005).

Recently, the mTOR kinase has emerged as a critical player in the regulation of Skp2 (Shapira et al., 2006). Rapamycin, which inhibits the mTOR by directly binding the mTORC1, significantly decreased the expression of Skp2 both at mRNA and protein levels in a dose and time-dependent manner in breast cancer cell lines (Shapira et al., 2006). Moreover, it was found that negative effect of rapamycin on Skp2 expression has a critical role in rapamycin-mediated cell growth and G1 arrest (Shapira et al., 2006). Furthermore, rapamycin promoted the degradation of Skp2 and down-regulated the expression of the APC/C inhibitor Emi1 in breast cancer (Shapira et al., 2006), indicating that Skp2 could be a novel target for mediating the effects of rapamycin; however, the molecular mechanism by which mTOR regulates Skp2 remains to be elucidated.

ERK AND ITS ROLE IN Skp2 SIGNALING

Multiple studies have shown that ERK activities are up-regulated in many human cancers including breast cancer, and elevated ERK activity in human tumors has been correlated with poor prognosis, demonstrating that ERK may play a crucial role in human tumorigenesis (Kohno and Pouyssegur, 2006). It is known that ERK family includes ERK1 and ERK2, which belong to MAPK super family that regulates cell cycle progression, cell proliferation, differentiation, survival, and apoptosis (Kolch, 2005). Activation of MAPK requires dual phosphorylation by specific MAPK kinases (MKKs), and dephosphorylation by protein phosphatases, including MAPK phosphatases (MKPs; also known as dual-specificity phosphatase, DUSP; Seger and Krebs, 1995). It is well accepted that many different stimuli such as growth factors or carcinogens could activate the ERK pathway, leading to the activation of its targeted proteins such as downstream kinases and transcription factors that regulates the expression of specific genes and their activators/modulators (Roberts and Der, 2007; Shaul and Seger, 2007).

Recently, it has been reported that Skp2 is involved in ERK pathway (Lin and Yang, 2006). Lin and Yang (2006) verified that Skp2 participated in the ERK-directed ubiquitination and proteolysis of MKP-1, suggesting that ERK activity could be controlled via MKP-1 proteolysis in cooperation with Skp2. Consistent with this notion, suppression of Skp2 expression resulted in DUSP-1 up-regulation in human hepatocellular carcinoma (Calvisi et al., 2008). Furthermore, the cooperation between ERK and Skp2 has also been found in human breast cancer whereby synergistic activity of the two oncogenes has been shown to increase p27 degradation (Foster et al., 2003). Moreover, Foster et al. (2003) showed that estrogens elicit down-regulation of p27 through Skp2-dependent and -independent mechanisms and could depend on p27 localization requiring the participation of other mediators of the Ras/Raf-1/ERK signaling pathway in breast cancer cells. However, it remains to be determined how Skp2 regulates the ERK activation in human breast cancers and the biological consequence of this regulation requires further investigation.

PI 3K/Akt IN RELATION TO THE Skp2 PATHWAY

The PI3K/Akt pathway has been reported to be involved in Skp2 pathway (Gao et al., 2009a). It has been well documented that Akt is a serine/threonine protein kinase, which is downstream of PI3K in response to growth factor stimulation (Hennessy et al., 2005). The Akt family of kinase includes three closely related isoforms designed as Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ. Activated Akt promotes cell survival by suppressing apoptosis through promoting the phosphorylation and subsequent cytoplasmic localization of many downstream pro-apoptotic protein targets including Bad and Forkhead transcription factors such as FOXO1 and FOXO3a (Hennessy et al., 2005). In addition, Akt also modulates apoptosis indirectly by influencing the activities of several transcription factors such as NF-κB and CEBP (cyclic AMP-responsive element binding protein; Franke, 2008). Enhanced Akt signaling can also promote cell growth by inhibition of negative cell cycle regulators p21 and p27 (Franke, 2008). Thus, Akt has been believed to be a promising target for cancer prevention and treatment (Fresno Vara et al., 2004).

It has been reported that PI3K/Akt regulates p27 expression via Skp2 in human cancer (Van Duijn and Trapman, 2006). Recently, we and other groups discovered that Akt controls Skp2 stability and the subcellular localization of Skp2 (Gao et al., 2009a; Lin et al., 2009). First of all, the positive correlation between Skp2 expression and Akt activity was found in a panel of breast cancer cell lines (Gao et al., 2009a). Moreover, inhibition of Akt1 activity in breast cancer cells caused down-regulation of Skp2 expression, indicating that elevated Akt activity could be one major cause of the observed up-regulation of Skp2 in breast cancer (Gao et al., 2009a). Further research toward exploration of the molecular mechanisms by which Akt promotes cytoplasmic localization of Skp2 still requires further attention.

IGF AND ITS CROSS-TALK WITH THE Skp2 PATHWAY

It is widely accepted that IGF-1 could bind to its receptors including IGF-1 receptor (IGF1R) and insulin receptor. In addition, IGF-1 has been shown to bind and interact with the IGFBPs (IGF-1 binding proteins), of which there are six identified members (IGFBP1-6) so far (Grimberg, 2003). After binding to the IGF1R, IGF-1 initiates cellular signaling such as activation of PI3K/Akt signaling pathway and/or Ras/Raf/MAPK pathway, which influences cell proliferation, survival, and apoptosis (Clayton et al., 2011). Therefore, the increased level of IGF-1 promotes cell proliferation, inhibits apoptosis, and enhances angiogenesis, all are involved in the development and progression of human cancers (Heidegger et al., 2011). A recent study has shown that IGF-1 regulates the expression of Skp2 in breast cancer (Lu et al., 2004). Lu and colleagues found that IGF-1 decreased p27 expression via up-regulation of Skp2. Moreover, chemotherapeutic drug trastuzumab (also known as Herceptin) decreased the expression level of Skp2, whereas this decrease was attenuated by IGF-1 in various breast cancer cells (Lu et al., 2004). Furthermore, IGF-1-mediated reduction in p27 protein mediated via increased Skp2 expression involves the activation of PI3K pathway, suggesting that

Skp2 could be an attractive target for the treatment of human cancer by attenuating multiple other growth signaling pathways.

PPARγ AND ITS RELATIONSHIP WITH Skp2

Peroxisome proliferator-activated receptor-γ, a ligand-activated transcription factor, has been demonstrated to provoke and mediate anti-inflammatory signaling (Schmidt et al., 2010). PPARγ binds to PPREs (peroxisome proliferators response elements) as a heterodimer with members of the RXR (retinoid X receptor) subfamily, leading to the regulation of target gene expression (Schmidt et al., 2010). It is widely accepted that PPARγ is a tumor suppressor protein because it initiates a number of anti-neoplastic processes such as arresting the cell cycle, causing cell differentiation, inhibition of angiogenesis as well as induction of apoptosis (Schmidt et al., 2010). It has been observed that PPARγ induced the expression of PTEN in breast cancer cell lines, possibly by binding to putative PPREs, resulting in Akt phosphorylation and reduced cell proliferation (Patel et al., 2001; Bonofiglio et al., 2005). However, one study demonstrated that down-regulation of PPARγ suppressed cell growth and induced apoptosis in MCF-7 breast cancer cells (Zaytseva et al., 2008), arguing that the tumor suppressive role of PPARγ could be cell-type or cellular context dependent.

Recently, PPARγ has been found to regulate Skp2 expression in human cancers including breast cancer (Wei et al., 2007; Meng et al., 2010). For example, thiazolidinediones (TZD), an agonist of PPARγ, have been found to down-regulate Skp2 with accumulation of its substrate p27 in prostate cancer (Wei et al., 2007). More recently, PPARγ overexpression was reported to suppress Skp2 levels. More importantly, the inverse correlation between the expression of PPARγ and Skp2 was identified in both breast cancer cell lines and human breast cancer specimens (Meng et al., 2010). Consistent with this, PPARγ overexpression inhibited the expression of Skp2 at both mRNA and protein levels, leading to reduced cell proliferation and induction of apoptosis (Meng et al., 2010). Furthermore, overexpression of Skp2 partially abrogates PPARγ's pro-apoptotic and anti-proliferative abilities (Meng et al., 2010), suggesting that anti-neoplastic role of PPARγ could in part be mediated through deregulation of Skp2 expression in breast cancer. However, more studies are required to fully understand how PPARγ regulates Skp2 signaling pathway in human breast cancer.

THE ROLE OF FoxP3 WITH RESPECT TO Skp2 SIGNALING

The FoxP3 (Forkhead box P3), an X-linked tumor suppressor gene, is a member of the forkhead box/winged-helix transcription factor family (Wang et al., 2010a). FoxP3 is highly expressed in regulatory T cells, behaving as a master regulator in the development and differentiation of regulatory T cells. In recent years, it has been shown that FoxP3 is frequently inactivated in many human cancers such as prostate cancer, ovarian cancer, and breast cancer (Zuo et al., 2007b; Wang et al., 2009; Zhang and Sun, 2010). For example, overexpression of FoxP3 inhibited cell proliferation, decreased cell migration, and reduced cell invasion in ovarian cancer (Zhang and Sun, 2010). In addition, FoxP3 retarded prostate tumorigenesis through inhibition of oncogene Myc (Wang et al., 2009). Moreover, FoxP3 has been identified as a transcriptional repressor of the HER2 oncogene (Zuo et al., 2007b). Interestingly, FoxP3 also suppressed growth and induced cell death in breast cancer cell line without HER2 overexpression (Zuo et al., 2007b), indicating that FoxP3 may affect other pathways that are involved in breast cancer. Indeed, Zhang et al. found that FoxP3 is a novel transcriptional repressor of Skp2 in human breast cancer (Zuo et al., 2007a). Overexpression of FoxP3 inhibited Skp2 expression with increased p27 in breast cancer cells, while down-regulation of FoxP3 in human mammary epithelial cells increased Skp2 levels (Zuo et al., 2007a). This finding has been further supported by the observed inverse correlation between FoxP3 and Skp2 levels in primary breast cancer samples (Zuo et al., 2007a). Moreover, down-regulation of Skp2 was critical for FoxP3-mediated growth inhibition in breast cancer cells (Zuo et al., 2007a), demonstrating that FoxP3 is a Skp2 repressor in breast cancer. However, how FoxP3 regulates Skp2 expression and whether this regulation could be exploited for fighting the battle against breast cancer will require further in-depth investigations.

Skp2 INHIBITION IS A NOVEL STRATEGY FOR BREAST CANCER TREATMENT

Since Skp2 is frequently amplified and overexpressed in human breast cancer, Skp2 could be a potential molecular target for breast cancer therapy (Ohta and Fukuda, 2004). Therefore, inhibition of Skp2 may be a novel strategy for the prevention and/or treatment of breast cancer. To this end, several small molecule inhibitors to block Skp2 expression have been developed by a high-throughput screening (Chen et al., 2008; Rico-Bautista et al., 2010). For example, a small molecular inhibitor CpdA (Compound A), which blocks the recruitment of Skp2 to the SCF ligase, caused cell cycle arrest, cell growth inhibition, and apoptosis in multiple myeloma cells (Chen et al., 2008). Moreover, one chemical compound known as SMIP0004 was found to down-regulate Skp2 and subsequently caused p27 stabilization in prostate cancer cells (Rico-Bautista et al., 2010). Unfortunately, specific drugs that inactivate Skp2 in breast cancer are unavailable so far, although we believe that there is renewed interest in developing Skp2 inhibitors for breast cancer treatment.

It is noteworthy that several natural compounds has been found to down-regulate Skp2 expression in human cancers including breast cancer (Huang et al., 2011). For example, curcumin, lycopene, pentagalloylglucose, and quercetin inhibited Skp2 expression, leading to cell growth inhibition and cell cycle arrest through increased FOXO1 in breast cancer cells (Huang et al., 2011). ATRA (all-trans retinoic acid) promoted the ubiquitination of Skp2 in breast cancer cell lines, leading to cell cycle arrest (Dow et al., 2001). Consistent with this notion, overexpression of Skp2 promotes resistance to ATRA and prevents p27 accumulation in breast cancer cells (Dow et al., 2001). Hsu et al. (2011) found that gallic acid markedly reduced cell growth of human breast cancer cells and induced cell cycle arrest by inhibition of Skp2 and attenuation of Skp2–p27 association as well as reduction of p27 ubiquitination. Huang et al. (2008) reported that EGCG (Epigallocatechin-3-gallate), the main constituent of green tea, inhibited human breast cancer cell growth in part through down-regulation of Skp2 expression and accumulation of p27. Furthermore, both tamoxifen and paclitaxel significantly and synergistically enhanced cell growth inhibition by EGCG mediated through the down-regulation of Skp2 expression in breast cancer

cells (Huang et al., 2008). Interestingly, the inhibition of Skp2 was not always correlated with increased p27 expression, indicating that EGCG-dependent Skp2 down-regulation could reduce cell growth via other downstream pathway(s) (Huang et al., 2008). These published data provides strong evidence in support of the idea that inhibition of Skp2 may be a promising therapeutic strategy for the treatment of human breast cancer.

CONCLUSION AND OVERALL PERSPECTIVES

In this short review article, we have provided succinct information as to the state of our knowledge on the role of Skp2 in human breast cancer. However, it is worth mentioning that the critical roles for Skp2 as an oncoprotein in human cancer progression are largely uncertain, although studies establishing the relationship between Skp2 and cancer have been burst onto the scene in recent years, and several groups have found that multiple genes including PI3K/Akt, ERK, mTOR, and FoxP3, could regulate the Skp2 expression (**Figure 2**). Skp2 has been revealed as a novel target for the prevention and/or treatment of human cancers including but not limited to breast cancer. Therefore, the development of agents for specifically targeting Skp2 is likely to have a significant therapeutic impact on the treatment of human cancers. Furthermore, here we summarized that several compounds, especially natural compounds that could specifically target Skp2 may act as anticancer drugs. Due to non-toxic features, inhibition of Skp2 by natural agents could be a novel and safer approach for empowering anticancer therapy. However, further pre-clinical studies are needed to find the right combinations with chemotherapeutic drugs toward better treatment of human mammary malignancies. We hope that this article could promote further study for the development of specific inhibitors/antagonists for targeted inactivation of Skp2 for cancer therapy by either single agent or by using a combinational approach. In conclusion, targeted inactivation of Skp2 would likely become a novel newer strategy for the prevention of tumor progression and/or successful treatment of human malignancies including breast cancer in the future.

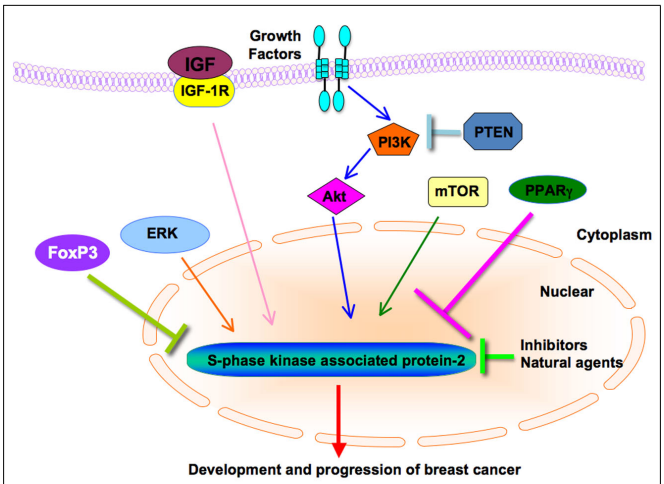

FIGURE 2 | Diagram of Skp2's cross-talks with other major signaling pathways in breast cancer. PI3K/Akt, mTOR, PPARγ, ERK, FoxP3, and IGF regulate the expression of Skp2 in the breast cancer. Chemical compounds including Skp2 inhibitors and natural agents inhibit cell growth and induce apoptosis through down-regulation of Skp2 expression in breast cancer. Skp2, S-phase kinase associated protein 2; ERK, extracellular signal-regulated kinase; IGF-1, insulin-like growth factor-1; FoxP3, forkhead box P3; mTOR, mammalian target of rapamycin; PI3K, phosphatidylinositol 3-kinase; PPARγ, peroxisome proliferator-activated receptor-γ.

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Regulation of ubiquitination-mediated protein degradation by survival kinases in cancer

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The ubiquitin–proteasome system is essential for multiple physiological processes via selective degradation of target proteins and has been shown to plays a critical role in human cancer. Activation of oncogenic factors and inhibition of tumor suppressors have been shown to be essential for cancer development, and protein ubiquitination has been linked to the regulation of oncogenic factors and tumor suppressors. Three kinases, AKT, extracellular signal-regulated kinase, and IκB kinase, we refer to as oncokinases, are activated in multiple human cancers. We and others have identified several key downstream targets that are commonly regulated by these oncokinases, some of which are regulated directly or indirectly via ubiquitin-mediated proteasome degradation, including FOXO3, β-catenin, myeloid cell leukemia-1, and Snail. In this review, we summarize these findings from our and other groups and discuss potential future studies and applications in the clinic.

Keywords: AKT, ERK, IKK, FOXO3, β-catenin, Mcl-1, snail

UBIQUITIN–PROTEASOME SYSTEM AND CANCER

In order to maintain cellular homeostasis, the amount of proteins in cells is selectively controlled not only in protein synthesis but also in protein degradation. The ubiquitin–proteasome pathway is essential for multiple physiological systems via selective degradation of target proteins (Hershko and Ciechanover, 1998). The proteins designated for proteasome-mediated degradation are conjugated with polypeptide of ubiquitin, which are then targeted to 26S proteasome complex (Hochstrasser, 1995). Ubiquitination of target protein is regulated through multi-enzyme processes in an ATP-dependent manner. First, the E1 protein, ubiquitin-activating enzyme, activates ubiquitin, which is then transferred to the E2 protein. The E3 protein is an ubiquitin protein ligase that determines the substrate specificity. The RING finger-containing E3 ligase binds to its substrate and the ubiquitinated E2 protein and then directly transfers the ubiquitin from the E2 protein to the substrate (Lipkowitz and Weissman, 2011). On the other hand, the HECT domain-containing E3 ligase can also receive ubiquitin from the E2 protein first through an active-site cysteine of its HECT domain then interacts with its substrate to catalyze the conjugation of the activated ubiquitin to the substrate (Kee and Huibregtse, 2007). Since the specificity of the target proteins for proteasome-mediated degradation is dependent on the interaction between the E3 ligases and their targets, the E3 ubiquitin ligases are critical for regulating the expression levels of key short-lived proteins.

Cancer is a genetic disease that is caused by multiple genetic mutations. In cancer cells, oncogenic drivers that are frequently mutated or overexpressed activate the signaling pathways to promote cell proliferation, growth, and survival while tumor suppressors that are commonly inactivated by mutation or deletion inhibit these pathways. It has been demonstrated that the expression levels of some key oncoproteins and tumor suppressors are under the control of ubiquitin–proteasome system with some E3 ligases that function as oncogenic factors or tumor suppressors. For example, MDM2 and Skp2 ubiquitinate and inhibit tumor suppressors via proteasomal degradation, and thereby function as oncogenic factors (Marine and Lozano, 2010; Wang et al., 2011). In contrast, other E3 ligases such as the anaphase promoting complex/cyclosome (APC/C) and F-box and WD repeat domain-containing 7 (FBW7) serve as tumor suppressors by downregulating oncogenic factors (Crusio et al., 2010; Wasch et al., 2010).

Accumulating evidence indicates that ubiquitin–proteasome pathways are potential drug targets for cancer therapy. For example, bortezomib is a specific proteasome inhibitor that is currently used for the treatment of multiple myeloma. Moreover, the inhibitors for specific E3 ligases have been also considered as potential anti-cancer drugs. MDM2 is the primary ubiquitin ligase for tumor suppressor protein, p53, which induces apoptosis or senescence in response to oncogenic stress or DNA damage. The p53 pathway is frequently inactivated in human cancer cells, and the small molecules that block the interaction between p53 and MDM2 to inhibit p53 degradation have been tested in clinical trials (Brown et al., 2009). Therefore, dissecting the specific ubiquitin–proteasome signaling facilitating cancer progression may contribute to the development of novel drug targets.

THREE SURVIVAL KINASES AND THEIR SIGNALING PATHWAYS

Protein kinases and phosphatases catalyze the protein phosphorylation and dephosphorylation, respectively, which are essential for maintaining signal transduction. When cells receive extracellular signaling and stress, the signals are primarily transduced to

the nucleus via protein phosphorylation, resulting in the alteration of gene expression. For example, epidermal growth factor (EGF) stimulates cell proliferation by binding to its receptor, EGF receptor (EGFR), and activating it. EGFR is a receptor tyrosine kinase that phosphorylates and activates multiple downstream targets and promotes cell growth and survival. EGFR, considered as an oncogene, is frequently overexpressed or mutated in multiple human cancers and promotes tumor progression, metastasis, and drug resistance (Nicholson et al., 2001; Hynes and Lane, 2005; Quatrale et al., 2011). Indeed, many oncoproteins include protein kinases, and these oncogenic kinases phosphorylate downstream targets to promote tumor growth, metastasis, and/or angiogenesis.

The serine/threonine kinase AKT is one of the major downstream kinases activated by growth factor signaling such as EGFR, platelet-derived growth factor receptor (PDGFR), and insulin-like growth factor receptor (IGFR). AKT has three isoforms, AKT1, AKT2, and AKT3, and their activities are frequently elevated in multiple human cancers, which contribute to cancer cell survival and growth (Altomare and Testa, 2005). AKT is activated by phosphatidylinositol-3 kinase (PI3K) that converts phosphatidylinositol (3,4)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) in plasma membrane through lipid phosphorylation (Altomare and Testa, 2005; Yuan and Cantley, 2008). AKT and phosphoinositide dependent kinase 1 (PDK1) are then recruited to the plasma membrane where AKT is directly phosphorylated and activated by PDK1 (Chan et al., 1999). Activated AKT phosphorylates various substrates involved in cell metabolism (GSK3, TSC2), survival (Bad, FOXO), and cell cycles (p21, p27, MDM2; Cross et al., 1995; Datta et al., 1997; Brunet et al., 1999; Zhou et al., 2001a,b; Liang et al., 2002; Manning et al., 2002), and then inhibits apoptosis and promotes cell growth. Therefore, the PI3K–AKT pathway is a potential drug target, and several PI3K or AKT inhibitors have been actively tested in numerous clinical trials (Garcia-Echeverria and Sellers, 2008; Wong et al., 2010; Chappell et al., 2011).

Extracellular signal-regulated kinase (ERK) is another critical downstream kinase in growth factor signaling and plays an essential role in cancer cell proliferation (Robinson and Cobb, 1997; Sebolt-Leopold, 2000). The Ras–Raf–MEK–ERK pathway is a well-characterized signaling pathway and is commonly activated in multiple human cancers. Studies have shown that Ras and Raf are frequently mutated in various human cancer types and responsible for cancer progression (Adjei, 2001; Davies et al., 2002). Therefore, this signaling pathway has been considered a drug target for cancer therapy, and a variety of inhibitors have been developed, including MEK or Raf inhibitors (Davies et al., 2007; Bollag et al., 2010; Hatzivassiliou et al., 2010; Maurer et al., 2011).

IκB kinases (IKKs) are the primary regulator of NF-κB, which plays a key role in immune response, cell proliferation, and survival (Baldwin, 2001; Luo et al., 2005). IKKs are activated in response to various cytokines and inflammatory stimuli such as tumor necrosis factor (TNF)-α, interleukin-1, and lipopolysaccharide. The IKK family includes IKKα, IKKβ, IKKγ, IKKε, and TANK-binding kinase 1 (TBK1). Interestingly, IKKγ has no kinase activity and functions as an adaptor protein for the canonical IKK complex (Hacker and Karin, 2006). IKKs are involved in two distinct pathways for NF-κB activation: the canonical and non-canonical pathways. IKKβ plays a dominant role in the canonical pathway and IKKα in the non-canonical pathway (Perkins, 2007; Israel, 2010). In the canonical pathway, IKKα, IKKβ, and IKKγ form a kinase complex that phosphorylates IκBα, an inhibitor protein of NF-κB, and induces the ubiquitination and subsequent proteasome-dependent degradation of IκBα. In the non-canonical pathway, IKKα forms a homodimer and phosphorylates p100, and generates p52 by partial processing of p100, resulting in the activation of p52/RelB. In contrast to IKKα and IKKβ, IKKε and TBK1 play a role in the induction of interferon signaling in response to viral infection (Shen and Hahn, 2011). Although NF-κB has been known to be involved in the progression of various cancers, increasing evidence suggests that IKKs also play vital roles in cancer independently of NF-κB (Lee and Hung, 2008; Baud and Karin, 2009; Shen and Hahn, 2011). For example, IKKα phosphorylates both estrogen receptor α (ERα) and co-activator SRC3 and enhances ERα transcriptional activity while IKKβ phosphorylates a tumor suppressor, tuberous sclerosis 1 (TSC1), and inhibits its function (Park et al., 2005; Lee et al., 2007). IKKα also phosphorylates CBP and the phosphorylated CBP preferentially interacts with NF-κB rather than p53, resulting in NF-κB activation as well as p53 inactivation (Huang et al., 2007). Recently, we found that IKKα phosphorylates and inhibits FOXA2. Inactivation of FOXA2 results in the decrease of NUMB expression, and subsequent NOTCH activation (Liu et al., 2012). Considering that inhibition of NF-κB may affect inflammatory responses, IKKs may be the potential alternative drug targets for cancer.

The above-mentioned three survival kinases play critical roles in cancer cell survival, metabolism, proliferation, and growth. Interestingly, these kinases have many common targets that they directly or indirectly regulate. Moreover, we and others have identified several common targets of these three kinases that are regulated by the ubiquitin–proteasome system. We will discuss these proteins one by one in the later next section of the review.

FORKHEAD BOX O

The forkhead box O (FOXO) family proteins are critical transcription factors that are involved in the regulation of cell proliferation, cell death, cell metabolism, and DNA repair (Tran et al., 2003; Arden, 2008). FOXO family includes FOXO1, FOXO3, FOXO4, and FOXO6, and is conserved from *C. elegans* to mammals (Burgering, 2008; Calnan and Brunet, 2008). FOXO family proteins directly activate multiple gene expression involved in cell cycles, apoptosis, metabolism, and DNA damage repair, such as p27kip, Bim, FasL, MnSOD, GADD45 (Dijkers et al., 2000; Kops et al., 2002; Tran et al., 2002; Ciechomska et al., 2003; Sunters et al., 2003). Moreover, it has been shown that FOXO proteins are dysregulated in multiple human cancers such as breast, prostate, leukemia, and glioblastoma (Hu et al., 2004; Seoane et al., 2004; Cornforth et al., 2008; Jagani et al., 2008). Conditional knockout mice of FOXO1, 3, and 4 develop thymic lymphomas and hemangiomas (Paik et al., 2007; Tothova et al., 2007). Therefore, FOXO is believed to function as a potential tumor suppressor.

It has been shown that IKK, AKT, and ERK directly phosphorylate FOXO and induce FOXO ubiquitination and degradation. Among the three kinases, AKT was first identified as a FOXO kinase that phosphorylates FOXO3 at T32, S253, and S315. Phosphorylated FOXO3 protein is excluded from entering the nucleus and binds to 14-3-3 in the cytoplasm (Brunet et al., 1999). In addition to FOXO3, FOXO1, and FOXO4 are also phoshorylated by AKT (Tzivion et al., 2011). AKT-phosphorylated FOXO1 and FOXO3 then undergo degradation in a proteasome-dependent manner (Plas and Thompson, 2003). Specifically, FOXO1 phosphorylated by AKT translocates to the cytosol where it is ubiquitinated by Skp2 and subjected to proteasome-dependent degradation (Huang et al., 2005). We found that IKKβ directly phosphorylates FOXO3 at S644 and induces its ubiquitination and degradation (Hu et al., 2004). Recently, E3 ligase β-transducing repeat-containing protein (β-TrCP) is reported to interact with FOXO3 and induces ubiquitination and degradation in an IKKβ-mediated-phosphorylation-dependent manner (Tsai et al., 2010; Su et al., 2011). Furthermore, we and others demonstrated that ERK phosphorylates FOXO3 and FOXO1, respectively (Asada et al., 2007; Yang et al., 2008). We also showed that FOXO3 is phosphorylated by ERK at S294, S344, and S425, which then undergoes MDM2-mediated ubiquitination, followed by proteasome-dependent degradation (Yang et al., 2008). MDM2-mediated ubiquitination and degradation is also observed with FOXO1, which is dependent on the AKT-mediated phosphorylation (Fu et al., 2009). Interestingly, MDM2 induces mono-ubiquitination of FOXO4, which promotes nuclear localization of FOXO4, and subsequent polyubiquitination by Skp2 and degradation (Brenkman et al., 2008). Taken together, the ubiquitin–proteasome system plays an essential role in regulating FOXO transcription factors by AKT, ERK, and IKK, and MDM2, SKP2, and βTrCP are E3 ligases for FOXO ubiquitination (**Figure 1A**).

β-CATENIN

β-Catenin is the key protein in both cadherin junction and Wnt pathway and plays an important role in development and adult homeostasis as well as tumorigenesis (Cadigan, 2008; Stepniak et al., 2009). In the Wnt signaling pathway, β-catenin functions as a transcription co-factor and is involved in the transactivation of several oncogenic proteins such as c-Myc, CyclinD1, and matrix metalloproteases (He et al., 1998; Lin et al., 2000; Mosimann et al., 2009). Glycogen synthase kinase-3β (GSK3β) and casein kinase 1 (CK1) are the major protein kinases regulating the β-catenin stability. In the absence of Wnt ligand, β-catenin forms a complex with Axin, APC, GSK3β, and CK1 and is phosphorylated by these kinases. Once phosphorylated, β-catenin undergoes β-TrCP-mediated ubiquitination and subsequent degradation. When Wnt binds to its receptor, Frizzles, and co-receptor, LRP5/6, the receptor complex recruits Axin–GSK3 complex to cell membrane, releasing β-catenin from the complex for its translocation to the nucleus where it activates gene transcription with T-cell factor (TCF) and lymphocyte enhancer factor (LEF).

AKT has been shown to directly phosphorylate GSK3β and inhibits it (Cross et al., 1995), and therefore, AKT seems to indirectly inhibit β-catenin degradation and inactivation through inhibition of GSK3β (Monick et al., 2001). In addition to the indirect mechanism, AKT directly mediates the β-catenin stability by phosphorylating β-catenin at S552. Once phosphorylated by AKT, β-catenin binds to 14-3-3ζ and is stabilized (Tian et al., 2004; Fang et al., 2007). In addition to AKT, we also demonstrated that ERK upregulates β-catenin via inhibition of GSK3β (Ding et al., 2005). Activated ERK directly interacts with GSK3β and phosphorylates it at T43. After the phosphorylation by ERK, GSK3 is primed for subsequent phosphorylation by p90RSK at S9, which inactivates it. Furthermore, IKK has been shown to be directly involved in the β-catenin regulation, and IKKα but not IKKβ phosphorylates

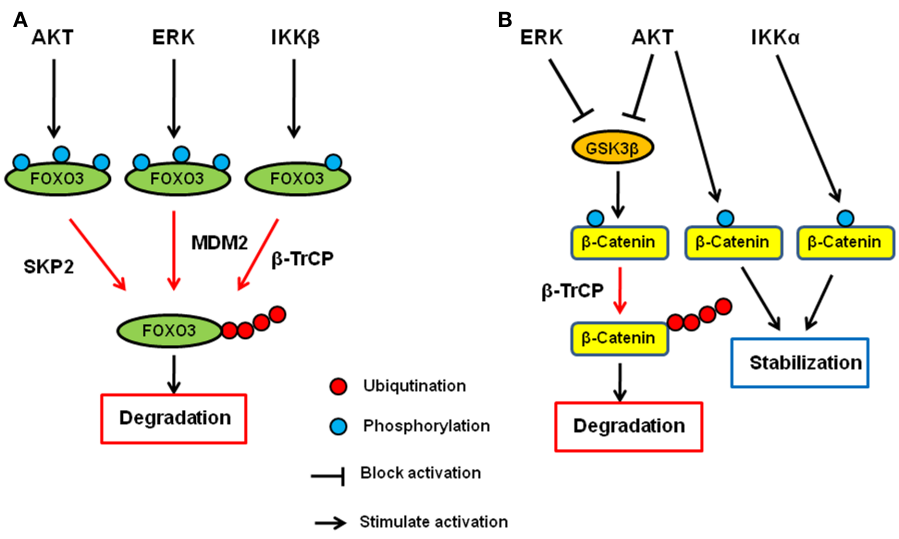

FIGURE 1 | Regulation of FOXO3 and β-catenin by AKT, ERK, and IKK signaling pathway. (A) AKT, ERK, and IKK phosphorylates FOXO3 at different sites and induces its ubiquitination and subsequent degradation via SKP2, MDM2, and β-TrCP, respectively. **(B)** AKT and ERK phosphorylates GSK3β and inhibit it. GSK3β phosphorylates catenin and induces its ubiquitination and subsequent degradation via β-TrCP. Both AKT and IKKα phosphorylate β-catenin and stabilize it.

β-catenin and prevent its ubiquitination and degradation (Lamberti et al., 2001; Albanese et al., 2003; Carayol and Wang, 2006). Collectively, β-catenin is another common target of AKT, ERK, and IKK, demonstrating that the ubiquitin–proteasome system plays a critical role in these signaling pathways (**Figure 1B**).

MYELOID CELL LEUKEMIA-1

Myeloid cell leukemia-1 (Mcl-1) is a member of anti-apoptotic Bcl-2 family proteins which are the central regulators of apoptosis signaling pathway (Inuzuka et al., 2011a). The Bcl-2 family consist of pro-apoptotic and anti-apoptotic proteins that regulate the release of apoptogenic proteins such as cytochrome *c* and Smac from mitochondria (Youle and Strasser, 2008). Pro-apoptotic Bcl-2 family proteins include the BH3-only proteins such as Bim, Bid, Bik, and Bad and multi-BH domain like Bax and Bak. Bax and Bak are able to form channels on the surface of mitochondria from which cytochrome *c* and Smac are released. Anti-apoptotic Bcl-2 family proteins, which include Bcl-2, Bcl-XL, and Mcl-1, prevent apoptotic cell death by inhibiting the activation and channel formation of Bax and Bak. In contrast, the BH3-only proteins directly bind to anti-apoptotic Bcl-2 family proteins and inhibit their functions. Thus, the balance of anti-apoptotic and pro-apoptotic proteins determines cell survival and death, and the expression of anti-apoptotic Bcl-2 family protein is the critical for cell survival. Among the anti-apoptotic Bcl-2 family proteins, Mcl-1 has a relatively short protein half-life (Nijhawan et al., 2003; Adams and Cooper, 2007) but its expression is enhanced in various cancer types (Placzek et al., 2010). Thus, Mcl-1 is an important survival factor in human cancer.

The primary kinase that regulates Mcl-1 stability is GSK3β. We and another group reported that GSK3 interacts with and phosphorylates Mcl-1 at S155, S159, and T163 (Maurer et al., 2006; Ding et al., 2007). In addition, phosphorylated Mcl-1 is ubiquitinated and undergoes proteasome-dependent degradation. So far, three possible ubiquitin ligases have been identified for Mcl-1. Mule is the BH3 domain-containing E3 ligase that was the first identified as the Mcl-1 ubiquitin ligase by biochemical purification (Zhong et al., 2005). Later, we found that β-TrCP can ubiquitinate and induce Mcl-1 degradation in GSK3β-mediated-phosphorylation-dependent manner (Ding et al., 2007). Recently, a tumor suppressor protein, FBW7, is shown to function as a Mcl-1 ubiquitin ligase (Inuzuka et al., 2011b; Wertz et al., 2011). FBW7-induced ubiquitination of Mcl-1 is also dependent the phosphorylation induced by GSK3β. Although both β-TrCP and FBW7 recognize the same GSK3β-mediated phosphorylation sites in Mcl-1, the exact roles of these two ubiquitin ligases under various apoptotic stresses are unknown.

AKT and ERK have been shown to upregulate Mcl-1 transcription (Wang et al., 1999; Booy et al., 2011). In addition, both PI3K/AKT and MEK/ERK pathways have also been shown to enhance Mcl-1 protein stability (Derouet et al., 2004). Because AKT inhibits GSK3β as described above, AKT increases Mcl-1 stability, at least in part, by inhibiting GSK3β. Indeed, the inhibition of PI3K induces S159 phosphorylation of Mcl-1 and subsequent ubiquitination and degradation of Mcl-1, which are blocked by GSK3 inhibition (Maurer et al., 2006). In addition to GSK3β, it has been show that ERK phosphorylates Mcl-1 at T92 and T163 (Domina et al., 2004; Ding et al., 2008). In particular, we showed that ERK-phoshorylated Mcl-1 can interact with Pin1 (Ding et al., 2008). Pin1 is a peptidyl–prolyl *cis*/*trans* isomerase that binds to specific pS/T-P motifs and then isomerizes its substrates, resulting in their conformational changes. Interestingly, Pin1 stabilizes Mcl-1 protein after the phosphorylation by ERK, and the expression of Mcl-1 correlates with Pin1 in multiple human cancer cell lines (Ding et al., 2008). Thus, ERK and Pin1 cooperatively regulate Mcl-1 stability. Regarding IKK, there is no evidence that IKK is directly involved in Mcl-1 or GSK3β phosphorylation and/or ubiquitination. However, it has been shown that NF-κB is required for EGF-induced Mcl-1 induction, suggesting that the IKK–NF-κB pathway plays a role in Mcl-1 expression or stability (Henson et al., 2003). Thus, Mcl-1 is a critical apoptosis regulator that is controlled by the three kinases at the post-translational as well as transcriptional level (**Figure 2A**).

SNAIL

Epithelial–mesenchymal transition (EMT) is an important physiological process that converts epithelial cells to mesenchymal cells which plays an essential role in embryonic development and tissue repair (Nieto, 2009). In addition, accumulating evidence shows that EMT is also critical for cancer metastasis. Epithelial cells lose cell–cell contacts and gain migratory properties during EMT. During cancer progression, cancer cells undergo EMT, resulting in increased motility, invasiveness, and aggressive behavior (Kalluri and Weinberg, 2009). Snail is a zinc-finger transcription repressor that is one of the EMT regulators. Snail family contains Snail1 (Snail), Snail2 (Slug), and Snail3 (Smuc; de Herreros et al., 2010). Like other EMT regulators, Snail suppresses E-cadherin expression by binding to its promoter. In addition to EMT, Snail is also involved in cell death, survival, stem cell, and immune regulation by controlling multiple target genes (Wu and Zhou, 2010). Downregulation of Snail reduces tumor growth and invasiveness in xenograft animal model (Olmeda et al., 2007). Moreover, Snail is overexpressed in multiple human cancers, and expression of Snail is associated with poor cancer prognosis (Peinado et al., 2007). Therefore, Snail is a critical oncogenic factor.

Snail stability is primarily regulated by GSK3β through the PI3K–AKT pathway (Song et al., 2009). We have shown that GSK3β can phosphorylate Snail at six serine residues in which two of them are responsible for Snail stability while the other four are involved in its nuclear localization (Zhou et al., 2004). First, GSK3β phosphorylates Snail at four serine residues to allow its export from the nucleus. Then, GSK3β phosphorylates the other two sites, resulting in β-TrCP-mediated ubiquitination and degradation of Snail. IKK–NF-κB pathway has also been shown to regulate Snail stability. Specifically, NF-κB induces COP9 signalosome 2 (CSN2), which disrupts the interaction between GSK3 and Snail, resulting in the inhibition of Snail ubiquitination and subsequent degradation (Wu et al., 2009). So far, there is no evidence to show that MEK–ERK signaling pathway is directly involved in Snail stability. However, ERK does play a role in c-Myc-induced EMT via the inhibition of GSK3β, and there by stabilizing Snail (Cho et al., 2010). Furthermore, ERK upregulates Snail gene transcription though activation of AP-1 transcription factor (Hudson et al., 2007; Li et al., 2010). Independently of kinases, wild type p53

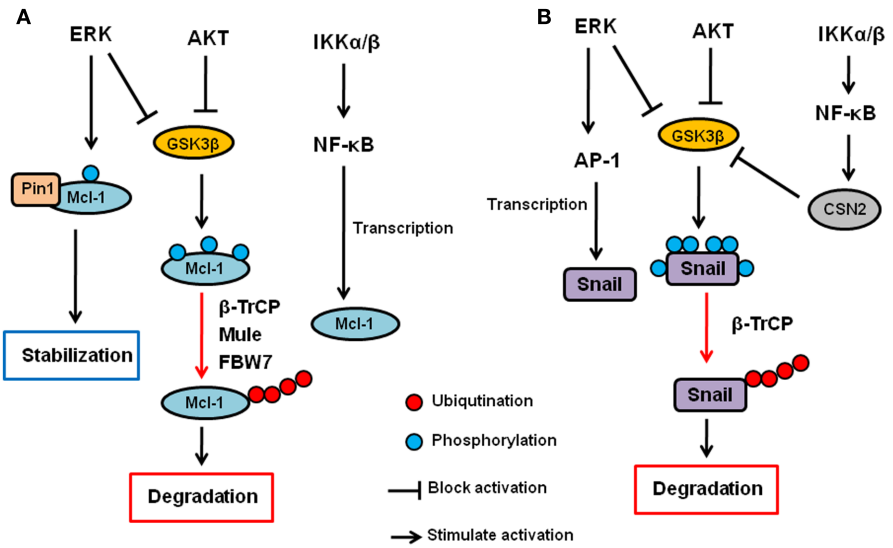

FIGURE 2 | Regulation of Mcl-1 and Snail by AKT, ERK, and IKK signaling pathway. (A) AKT and ERK phosphorylates GSK3β and inhibit it. GSK3β phosphorylates Mcl-1 and induces its ubiquitination and subsequent degradation via β-TrCP, Mule, and/or FBW7. ERK also directly phosphorylates Mcl-1, and phosphorylated Mcl-1 interacts with Pin1, resulting in its stabilization. The IKK–NF-κB pathway also contributes to Mcl-1 upregulation but the mechanisms are uncertain. **(B)** AKT and ERK phosphorylates GSK3β and inhibit it. GSK3β phosphorylates Snail and induces its nuclear exclusion, ubiquitination and subsequent degradation via β-TrCP. ERK also upregulates Snail transcription via AP-1 transcription factor. The IKK–NF-κB pathway inhibits Snail via upregulation of CSN2, which interferes with the GSK3–Snail interaction and ubiquitination of Snail.

but not mutant p53 has been shown to interact with and induce Snail and Slug ubiquitination and degradation (Wang et al., 2009; Lim et al., 2010). MDM2 functions as an ubiquitin ligase for p53-induced Snail ubiquitination. Moreover, F-box and leucine-rich repeat protein 14 (FBXL14) has been shown to interact with and induce Snail ubiquitination and degradation (Vinas-Castells et al., 2010). So far, no other kinases have been reported to be involved in MDM2- or FBXL-mediated Snail ubiquitination. Thus, the three kinases (AKT, ERK, and IKK) seem to be involved in EMT, at least in part, by regulating Snail expression through ubiquitination (**Figure 2B**).

CONCLUSION AND FUTURE PROSPECT

Recent advances in signal transduction studies have identified many key oncogenic kinases and their substrates in cancer progression, and the signaling pathways associated with these kinases have been recognized as promising drug targets. Indeed, several kinase inhibitors have been developed and used in clinic that show high efficacy and low toxicity (Sharma and Settleman, 2010). However, several clinical studies have emerged showing that some patients exhibit little or no response to these targeted drugs, and those who originally responded the drugs eventually developed resistance. Although the detailed mechanisms underlying drug resistance are not fully understood, there is evidence to support that alternative pathways are being activated in resistant cells to compensate for the survival signal blocked by targeting agents. For example, EGFR tyrosine kinase inhibitors (TKIs) are effective drugs for EGFR mutant lung cancer, but c-MET amplification or K-Ras mutation causes the resistance to TKIs by bypassing the inhibition of survival signaling (Bean et al., 2007; Linardou et al., 2008). We also showed that cancer cells with high AKT signaling pathway exhibit resistance to ERK inhibitors by inhibiting FOXO3 (Yang et al., 2010). Therefore, in order to develop effective personalized cancer therapy, it would be essential that we understand the cross-talk among the multiple oncogenic signaling pathways.

In this review, we introduced AKT, ERK, and IKK as the key survival kinases for cancer progression and survival. We also mentioned that these three pathways have several common targets that are critical for cancer cell proliferation, survival, and EMT. Because there are many other pathways that are activated in human cancers, we believe that they likely also contribute to the same targets we described here. These three kinase-signaling pathways may have other common targets that are critical for cancer progression. We also introduce some examples of the signaling pathways that are controlled via the ubiquitin–proteasome system, which seems to play an essential role in signaling pathway like phosphorylation. As we described above, inhibition of protein ubiquitination has been demonstrated to be potential drug targets for cancer therapy. Moreover, blockade of specific ubiquitination may exhibit less toxicity because inhibition of upstream molecules in key oncogenic signaling pathways may affect numerous signaling pathways and induce unfavorable side effects. Clearly, further studies for signaling pathways in cancer including post-translational modifications are required for the development of effective personalized cancer therapies.

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The APC/C ubiquitin ligase: from cell biology to tumorigenesis

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The ubiquitin proteasome system (UPS) is required for normal cell proliferation, vertebrate development, and cancer cell transformation. The UPS consists of multiple proteins that work in concert to target a protein for degradation via the 26S proteasome. Chains of an 8.5-kDa protein called ubiquitin are attached to substrates, thus allowing recognition by the 26S proteasome. Enzymes called ubiquitin ligases or E3s mediate specific attachment to substrates. Although there are over 600 different ubiquitin ligases, the Skp1–Cullin–F-box (SCF) complexes and the anaphase promoting complex/cyclosome (APC/C) are the most studied. SCF involvement in cancer has been known for some time while APC/C's cancer role has recently emerged. In this review we will discuss the importance of APC/C to normal cell proliferation and development, underscoring its possible contribution to transformation. We will also examine the hypothesis that modulating a specific interaction of the APC/C may be therapeutically attractive in specific cancer subtypes. Finally, given that the APC/C pathway is relatively new as a cancer target, therapeutic interventions affecting APC/C activity may be beneficial in cancers that are resistant to classical chemotherapy.

Keywords: ubiquitin, cell cycle, differentiation, cancer, ubiquitin ligase, cancer therapy

INTRODUCTION

Any discussion of the anaphase promoting complex/cyclosome (APC/C) pathway as a possible therapeutic target has to start with the question of what makes APC/C unique among the many ubiquitin ligases present in human cells. On one level APC/C is mechanistically similar to the Skp1–Cullin–F-box (SCF) ubiquitin ligases where there are core subunits and an adaptor protein that directly binds to a substrate. In SCFs, the four subunits are Skp1 (scaffold protein), Cul1 (scaffold protein), RING-finger component (Rbx1), and the variable adaptor protein or F-box protein that recognizes substrates (Zheng et al., 2002). In APC/C there are 13 subunits as well as variable adaptor proteins termed Cdc20 (or Fizzy; Fzy), Cdh1 (or Fizzy-related; Fzr), Cortex, Ama1, or Mfr1 (Acquaviva and Pines, 2006; Hutchins et al., 2010; Kops et al., 2010). However, beyond these similarities regarding adaptors and subunits present in APC/C and SCF ligases, large differences in size and structure exist between these two types of ligases. By contrast to SCF ubiquitin ligases, there is only limited knowledge regarding APC/C structure mainly because the enormous size and complexity of the holoenzyme present significant challenges for structure determination at the atomic level. The first structural insights into the APC/C was obtained by cryo-EM of complexes purified from human cells, *Xenopus laevis* egg extracts, and budding yeast (Gieffers et al., 2001; Dube et al., 2005; Passmore et al., 2005). 3D modeling showed that in all cases the APC/C is an asymmetric triangular complex (200 by 230 Å in size), composed of an outer wall and an internal cavity. Cdh1 and the Cullin domain of the Apc2 subunit are located on the outside of the complex, making it plausible that ubiquitination reactions occur on the outside and not inside the cavity. An emerging view of the APC/C is that of a four-part enzyme composed of a structural arm or scaffolding unit made of Apc1, Apc4, and Apc5, a catalytic arm consisting of Apc2, Apc11, and Doc1 (or Apc10), a tetratricopeptide repeat (TPR) arm made of Cdc23, Cdc16, and Cdc27, which mediates binding to activators and coactivators (Cdc20, Cdh1, Cortex). Other subunits such as Cdc26, Apc9, and Swm1 stabilize the TPR arm (Schwickart et al., 2004; Thornton and Toczyski, 2006). The TPR subunits have 12–15 copies of the 34-amino acid long TPR. They facilitate interactions between subunits and the assembly of multisubunit complexes (Zachariae and Nasmyth, 1999). A pseudo-atomic model of the yeast APC/C obtained by reconstitution studies of the holoenzyme and its subcomplexes has revealed that the TPR arm along with the structural

Abbreviations: APC/C, anaphase promoting complex/cyclosome; bHLH, basic helix–loop–helix; BMP, bone morphogenetic protein; Cdc20, cell division cycle protein 20 or Fizzy, Fzy; Cdh1, Cdc20 homolog 1, or Fizzy-related, Fzr; Cdk, cyclin-dependent kinase; CK1, casein kinase 1; D-boxes, destruction boxes; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligases; Emi1, early mitotic inhibitor-1/Rca1; HAT, histone acetyltransferase; HDAC, histone deacetylase; HEC1, high expression in cancer 1; Id1, inhibitor of DNA binding 1; Id2, inhibitor of DNA binding 2, also known as inhibitor of differentiation 2; JNK, c-Jun NH2-terminal kinase; MAK, male germ cell-associated kinase; MCM, minichromosome maintenance; MEF, mouse embryonic fibroblast; MR, methionine–arginine; NAALADase, *N*-acetylated alpha-linked acidic peptidase; Nek2, NIMA-related kinase 2; NGF, nerve growth factor; OSCC, oral squamous cell carcinomas; PC, proteasome/cyclosome; PCNA, proliferating cell nuclear antigen; PIP3, phosphoinositide-3,4,5-triphosphate; Plk1, polo-like kinase 1; PMSA, prostate specific membrane antigen; pRb, retinoblastoma protein; preRC, pre-replicative complex; Rbx1, RING-finger component; SCF, Skp1–Cullin–F-box; TAME, tosyl-L-arginine methyl ester; TGF-β, transforming growth factor beta; TPR, tetratricopeptide repeat; UPS, ubiquitin proteasome system.

arm coordinate the juxtaposition of the catalytic arm and the TPR phosphorylation sites relative to the coactivators, substrates, and regulators (Schreiber et al., 2011).

A previously unidentified APC/C subunit, Apc16, was reported recently (Hutchins et al., 2010; Kops et al., 2010). It is a small protein of 11.7 kDa in size encoded by Chromosome 10 open reading frame 104 (C10orf104) in humans. Apc16 may facilitate Cdc27 hyperphosphorylation, although it is not essential for assembly of the holocomplex (Kops et al., 2010). Therefore, the APC/C is composed of multiple subunits, some of which are newly discovered. However, the minimum ubiquitin ligase module of the APC/C that can catalyze ubiquitination is comprised of just two subunits – the Apc2 Cullin subunit and the Apc11 RING subunit (Gmachl et al., 2000; Leverson et al., 2000; Tang et al., 2001), which are analogous to the Cullin and Rbx1 subunits of the SCF complex (Barford, 2011). Thus, while we still do not have a complete understanding of APC/C structure, we are beginning to understand the general architecture of the complex, and possibly achieve an atomic level resolution of APC/C subcomplexes. These subcomplexes may provide multiple binding sites for small molecules that would perhaps make APC/C unique among ubiquitin ligases as a therapeutic target.

APC/C ACTIVITY

Another attractive aspect of the APC/C as a drug target is that it binds a unique set of enzymes required for transferring ubiquitin to substrates. The process of ubiquitination begins with the ubiquitin-activating enzyme E1 binding to and activating ubiquitin in an ATP-dependent manner. This activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme or E2. The ubiquitin ligases or E3 enzymes then associate with E2s to catalyze the ubiquitin transfer to the ε-amino group of lysine residues on substrate proteins (Ye and Rape, 2009). Multiple ubiquitin molecules can be linked together in different ways to form polyubiquitin chains that satisfy different objectives. In yeast, chains linked via Lysine 48 of ubiquitin (K48 chains) are a "proteolytic signal" whereas those linked via Lysine 63 (K63 chains) function as molecular scaffolds. In higher eukaryotes, the APC/C is known to build atypical K11-linked polyubiquitin chains on its substrates in association with its unique E2 partner, Ube2C (or UbcH10; Wickliffe et al., 2011b). Ube2C only initiates chain formation, however. Chain elongation is carried out by a K11-specific E2 called Ube2S (or E2–EPF) that works with both APC/C^{Cdc20} and APC/C^{Cdh1} (Garnett et al., 2009; Wu et al., 2010; Wickliffe et al., 2011a). The importance of this dual regulation of APC/C activity via Ubch10 and Ube2S is underscored by the finding that removing K11-specific E2s causes defects in spindle assembly and mitotic progression (Williamson et al., 2009).

One of the most attractive means of attenuating APC/C activity pharmacologically is by modulating the adaptor protein–substrate binding reaction. Early studies in yeast identified the Cdc20 and Cdh1 substrate binding adaptor proteins as required for APC/C activity (Visintin et al., 1997). Subsequent studies in multiple experimental systems demonstrated the biochemical requirements for these adaptors to bind their substrates (Fang et al., 1998). Although exceptions exist, Cdc20 and Cdh1 bind substrates containing the sequence elements RXXLXXXXN/D/E or destruction boxes (D-boxes), while Cdh1 can also bind substrates containing KEN sequences (Glotzer et al., 1991; Pfleger and Kirschner, 2000). Substrate binding initiates ubiquitination mediated by APC/C^{Cdc20} or APC/C^{Cdh1}. However, APC/C^{Cdc20} or APC/C^{Cdh1} mediated substrate binding is controlled during the cell cycle using an overlapping series of regulatory mechanisms. Inhibitory complexes control APC/C activity during the cell cycle, thus limiting its activity to defined temporal windows (for reviews see Manchado et al., 2010; Qiao et al., 2010; Pines, 2011). One of the most important examples of this is the exquisite control of APC/C^{Cdc20} activity during mitosis. In early mitosis, APC/C^{Cdc20} activity is curtailed by the spindle assembly checkpoint (SAC), which monitors the attachment of kinetochores to the mitotic spindle. An inhibitory complex containing Mad2, Bub3, and BubR1 proteins sequesters Cdc20 and renders it unable to bind substrates (Kim and Yu, 2011). After the checkpoint is switched off, this complex is released from APC/C^{Cdc20}, which initiates the metaphase to anaphase transition by mediating degradation of key proteins such as securin, shugoshin, and cyclin B1 (Kim and Yu, 2011). Securin and cyclin B1 degradation activates separase, which cleaves the cohesin complex that holds sister chromatids together while shugoshin destruction relieves sister chromatid cohesion at the centromere (Wang and Dai, 2005). These reactions are essential for the metaphase to anaphase transition, which is inhibited when APC/C activity is abrogated pharmacologically or by siRNA depletion or genetic disruption of *Cdc20* (Huang et al., 2009; Manchado et al., 2010; Zeng et al., 2010). Cells lacking APC/C^{Cdc20} eventually undergo mitotic catastrophe and die in a manner reminiscent of microtubule inhibition (Zeng et al., 2010). However, APC/C inhibitory molecules may have the added advantage that they will not have the usual off-target effects of microtubule inhibition well-known during chemotherapy treatment (Huang et al., 2009; Manchado et al., 2010).

APC/C^{Cdh1} CONTROLS SEVERAL CELL CYCLE TRANSITIONS

Small molecules that inhibit substrate ubiquitination via APC/C^{Cdc20} also reduce APC/C^{Cdh1} activity (Zeng et al., 2010). Since the APC/C^{Cdh1} window of activity during the cell cycle is larger than that of APC/C^{Cdc20}, it may be easier to modulate APC/C^{Cdh1} specific pathways necessary for growth of a particular cancer cell. APC/C^{Cdh1} controls mitotic exit and maintains the G1-phase in cycling cells (**Figure 1**). Outside the cell cycle, APC/C^{Cdh1} is required for quiescence, cell cycle exit, and differentiation (**Figure 1**). These multiple roles suggest the presence of distinct upstream regulatory and signaling pathways controlling APC/C^{Cdh1} within temporal or developmental windows. Importantly, these pathways will likely provide unique interactions with APC/C that can be targeted pharmacologically.

APC/C^{Cdh1} IS REQUIRED FOR MITOTIC EXIT AND G1 MAINTENANCE

APC/C^{Cdh1} targets multiple substrates for degradation during mitosis (**Figure 1**). Foremost among these are the mitotic cyclins, whose degradation ablates cyclin-dependent kinase 1 (CDK1) activity (Brandeis and Hunt, 1996; Irniger and Nasmyth, 1997). APC/C also reduces activity of multiple CDKs by initiating degradation of two components of the SCF ubiquitin ligase, the F-box protein Skp2 and an accessory protein, Cks1 (Bashir et al., 2004;

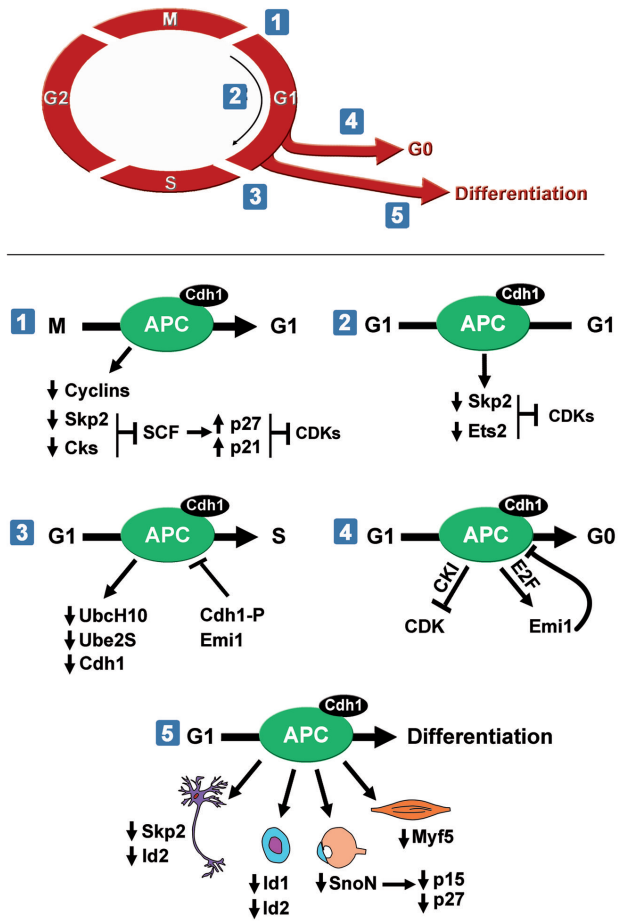

FIGURE 1 | APC/C^{Cdh1} regulates several cell cycle phases. (1, 2) APC/C^{Cdh1} promotes mitotic exit and maintains G1-phase by inhibiting CDK activity. (3) APC/C^{Cdh1} needs to be inactivated for cells to enter S phase. APC/C^{Cdh1} promotes cell cycle exit to a quiescent state (4) or differentiation (5). APC/C^{Cdh1} induces differentiation of neurons, hematopoietic cells, lens cells, and myocytes.

Wei et al., 2004). Degradation of these two substrates raises the levels of the cyclin-dependent kinase inhibitors p27^{Kip1} and p21^{Cip1}, which are normally targeted for degradation in an SCF^{Skp2} and Cks1 dependent manner (Ganoth et al., 2001; Bornstein et al., 2003). CDK inhibitors associate with specific cyclins and CDKs, preventing them from binding to ATP, and hence blocking their catalytic activity. Thus, high p27^{Kip1} and p21^{Cip1} levels maintain an early G1 state by reducing CDK activity. Another mechanism to promote G1 by reducing CDK activity involves APC/C^{Cdh1} mediated inhibition of an activator of S phase entry, cyclin D1. APC/C^{Cdh1} initiates degradation of the transcription factor Ets2 (Li et al., 2008), which normally controls cyclin D1 levels (Albanese et al., 1995). Consistent with these findings, depletion of Cdh1 by siRNA stabilizes Skp2 and Ets2, resulting in p21^{Cip1} and p27^{Kip1} degradation and cyclin D1 elevation in G1, followed by premature S phase entry (Wei et al., 2004; Li et al., 2008) and increased proliferation (Bashir et al., 2004). Thus, APC/C^{Cdh1} function during mitotic exit is coupled to G1 maintenance. It initiates a sharp decrease in CDK1 activity during mitotic exit by targeting the mitotic cyclins for destruction and subsequently remains active in early G1 to ensure that they remain inactive. In addition, it ensures that other cyclin-dependent kinases, namely CDK2/cyclin E, CDK2/cyclin A, CDK4/cyclin D, and CDK6/cyclin D are inactive since it maintains high p27^{Kip1} levels and low cyclin D1 levels through Skp2 and Ets2 degradation.

One question that immediately arises from these studies is that if APC/C keeps CDKs inactive in early G1, how do cells eventually reach S phase. The answer lies in the multiple mechanisms that decrease APC activity (**Figure 1**). For instance, the ubiquitination of the APC/C-specific ubiquitin-conjugating enzyme (E2) UbcH10 by APC/C^{Cdh1} provides a negative feedback mechanism that eventually dampens APC/C^{Cdh1} activity (Rape and Kirschner, 2004; Rape et al., 2006). Further, Cdh1 is inactivated by both phosphorylation and degradation (Lukas et al., 1999; Listovsky et al., 2004; Benmaamar and Pagano, 2005). Finally, E2F activates the transcription of the APC/C pseudo-substrate early mitotic inhibitor-1 (Emi1)/Rca1 in late G1, which inhibits APC/C^{Cdh1} activity (Hsu et al., 2002). These distinct mechanisms ensure that APC/C^{Cdh1} activity remains low from late G1 until the subsequent metaphase when it is activated via reduction of CDK and Emi1 activity (Kotani et al., 1998; Hsu et al., 2002; **Figure 1**).

APC/C^{Cdh1} CONTROLS PRE-REPLICATION COMPLEX FORMATION

One of the main reasons APC/C^{Cdh1} activity must remain low during S phase is that it is an inhibitor of pre-replication complex formation required for S phase entry (Diffley, 2004). APC/C^{Cdh1} controls the formation of the pre-replication complexes by modulating levels of three major regulators of the formation of the complex: Orc1, Cdc6, and geminin. For DNA replication to proceed, cells need to alternate between periods of low CDK activity and low geminin levels, in which the pre-replicative complexes (preRCs) are assembled; and periods of high CDK activity and high geminin levels in which origin firing and DNA replication occurs (McGarry and Kirschner, 1998; Petersen et al., 2000; Araki et al., 2003). APC/C^{Cdh1} is crucial to properly regulate the switch between these two states. For instance, Orc1 and Cdc6 are degraded via APC/C^{Cdh1} during early G1 (Petersen et al., 2000; Araki et al., 2003). Further, geminin levels are tightly controlled by APC/C^{Cdh1}. Geminin prohibits initiation of DNA replication at inappropriate times of the cell cycle by preventing MCM recruitment at the replication origins. During G1 the APC/C is active and as a consequence, geminin concentration is low. At the G1–S transition, APC/C is inactivated and geminin begins to accumulate. However, geminin concentration is not sufficient to inhibit a first wave of preRC formation and DNA replication begins. As S phase progresses, geminin accumulates and inhibits subsequent recruitment of MCMs to the replication origins (McGarry and Kirschner, 1998), and therefore re-duplication is avoided (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000).

APC/C^{Cdh1} PROMOTES CELL CYCLE EXIT AND QUIESCENCE

Multiple studies suggest that a major APC/C^{Cdh1} function outside of the cell cycle is limiting CDK activity required for cell cycle progression (Qiao et al., 2010). APC/C^{Cdh1} regulates CDK inhibitors that reduce CDK activity and initiate cell cycle exit. Adding a

second layer of regulation to APC/C^{Cdh1} activity, Binné et al. (2007) connected retinoblastoma protein (pRb) to APC/C^{Cdh1}, Skp2, and CDK inhibitor dependent cell cycle exit. These studies demonstrated that hypophosphorylated pRb associates with the APC/C specifically when activated by Cdh1, thus promoting Skp2 degradation and accumulation of p27^{Kip1} and p21^{Cip1}. This important finding linked extracellular signaling mechanisms, which normally control pRb activity to APC/C dependent degradation of Skp2 and initiation of cell cycle exit.

Once cells have exited the cell cycle, APC/C activity is required to maintain quiescence or differentiation. For instance, APC^{Cdh1} inactivation by deleting its Apc2 subunit in adult hepatocytes induced these otherwise quiescent cells to re-enter the cell cycle (Wirth et al., 2004). Similarly, APC^{Cdh1} has also been proposed to block postmitotic differentiated neurons from inappropriate cycling and apoptosis (Almeida et al., 2005; Jackson, 2006).

APC/C^{Cdh1} REGULATES CELL CYCLE EXIT AND CELL DIFFERENTIATION

APC/C^{Cdh1} activity promotes cell cycle exit and differentiation since Cdh1 depletion reduces differentiation of muscle, lens, hematopoietic, and neuronal cells (Lasorella and Iavarone, 2006; Li et al., 2007; Wu et al., 2007; Garcia-Higuera et al., 2008). Although our knowledge of the involvement of APC/C^{Cdh1} in the differentiation of other tissues awaits further investigation, its critical function in the degradation of cell cycle proteins suggests it likely plays relevant roles in linking quiescence and differentiation in most cell types. Further, since cancer progression is often thought to involve a dedifferentiation process (Daley, 2008; Trosko, 2009), understanding APC/C's involvement in normal cell cycle exit and differentiation will give us clues as to how APC/C regulation or substrate targeting may be misregulated during tumorigenesis.

Neuronal differentiation

Following a period of proliferation, neural progenitors differentiate into postmitotic neurons. Since Cdh1 levels are (Gieffers et al., 1999; Stegmuller and Bonni, 2005) higher in postmitotic neurons relative to their neural progenitors (Yao et al., 2010), APC/C^{Cdh1} may play a role in neuronal cell cycle exit. Consistent with this notion, an increase in APC/C^{Cdh1} activity and a decrease of APC/C^{Cdh1} substrates has been observed during terminal differentiation (Almeida et al., 2005; Yao et al., 2010). By contrast, upregulation of APC/C^{Cdh1} substrates has been described in human neural tumors, suggesting that APC/C^{Cdh1} activity is attenuated under these conditions (Lasorella and Iavarone, 2006; Eckerle et al., 2009).

Inhibitor of DNA binding 2

One of the targets that couples APC/C^{Cdh1} activity with neuronal differentiation is inhibitor of DNA binding 2 (Id2, also known as inhibitor of differentiation 2). In the developing nervous system, Id2 has been shown to inhibit the activity of neurogenic basic helix–loop–helix (bHLH) transcription factors required for neuronal differentiation (Yokota, 2001; Perk et al., 2005; Rothschild et al., 2006; Jung et al., 2010). APC/C^{Cdh1} targets Id2 for destruction, which couples cell cycle exit, differentiation, and axonal growth during the differentiation of diverse neuronal types (Lasorella and Iavarone, 2006; Yao et al., 2010). Thus, Id2 protein downregulation via proteolysis is essential for neuronal differentiation, suggesting that deregulation of Id2 destruction may underlie neural tumors.

Deregulated Id2 expression prevents cell cycle arrest via a wide range of signals (Lasorella et al., 2002; Kowanetz et al., 2004; Baghdoyan et al., 2005). Id2 downregulation via degradation allows the bHLH transcription factor E47 levels to increase and subsequently promote neural cell differentiation through induction of the CDK inhibitor p57^{Kip2} in the developing mouse brain (Rothschild et al., 2006; Tury et al., 2011). Similarly, decreasing Id2 levels increases expression of known mediators of neuronal differentiation such as Hes1 and Ascl1 (Mash1) transcription factors (Havrda et al., 2008) and NeuroD/E47 (Jung et al., 2010). Under certain experimental conditions, ectopic Id2 is able to drive terminally differentiated cells back into the cell cycle (Chaudhary et al., 2005). Importantly, the observation that the most aggressive tumors frequently contain the highest levels of Id proteins raises the possibility that deregulating Id protein stability by APC/C^{Cdh1} might also contribute to Id accumulation in cancer.

Skp2

The APC/C substrate Skp2 controls the G1 to S transition by eliminating numerous regulatory proteins that inhibit S phase entry (Reed, 2008). The SCF^{Skp2} target p27^{Kip1} plays a large role in cell cycle exit and differentiation, particularly neuronal differentiation (Durand et al., 1998; Vernon et al., 2003; Tarui et al., 2005; Nguyen et al., 2006, 2007). Genetic disruption of *p27^{Kip1}* causes a general increase in cell proliferation (Fero et al., 1996; Carruthers et al., 2003), including neurogenesis in the cortex and spinal cord (Nguyen et al., 2007; Li et al., 2009). Control of p27^{Kip1} is dual, subject to the accumulation of both cyclin-CDK complexes, which promote its phosphorylation, and Skp2, which promote the ubiquitination of phosphorylated p27^{Kip1} (Carrano et al., 1999; Montagnoli et al., 1999). Harmey et al. (2009) described an essential role for APC/C^{Cdh1} in cerebellar granule progenitors by mediating Skp2 destruction, thus coordinating cell cycle exit and terminal differentiation. Similar studies uncovered APC/C^{Cdh1}–Skp2 dependent differentiation of human embryonic stem cells (Bar-On et al., 2010). Collectively, these data suggested that two well-known inducers of neuronal differentiation, nerve growth factor (NGF), and retinoic acid, hyperactivate APC/C^{Cdh1}. NGF rapidly induced APC/C^{Cdh1} activity and promoted degradation of APC substrates, including cyclin B1 and the F-box protein Skp2 (Harmey et al., 2009). Similarly, retinoic acid promoted neuronal differentiation through increasing APC/C^{Cdh1} activity (Cuende et al., 2008; Yao et al., 2010). Further, retinoic acid induced nuclear accumulation of Cdh1, enhancing APC/C^{Cdh1} activity, Skp2 destabilization, and p27^{Kip1} accumulation (Cuende et al., 2008) by reducing expression of Rae1, a nuclear export factor that limits APC/C^{Cdh1} activity in mitosis (Yao et al., 2010). Similar to NGF and retinoic acid, bone morphogenetic protein 2 (BMP2) promotes cell cycle arrest via downregulation of Skp2 and accumulation of p27^{Kip1} in a neuroblastoma cell line (Nakamura et al., 2003). Thus, Skp2 degradation via APC/C^{Cdh1} is linked to neuronal differentiation and various signaling pathways. Disruption of signaling pathways controlling the APC/C–Skp2 axis during neuronal differentiation may lead to disruption of homeostasis. Consistent with this

possibility, reduced Skp2 and cyclin B1 expression has been seen in a transgenic model of Down syndrome where alteration of cell cycle rate and reduction of neurogenesis in the cerebellum was described (Contestabile et al., 2009). Moreover, *Skp2* transcript levels gradually increase with the aggressiveness of neuroblastoma subtype (Westermann et al., 2007), making the regulation of Skp2 by APC/C^{Cdh1} an attractive target in tumorigenesis.

Muscle cell differentiation

Myogenesis is a multistep process that sequentially requires the proliferation of committed myoblasts, the differentiation of myoblasts into postmitotic myocytes, and finally fusion of myocytes to form a multinucleated myotube with contractile capability. In muscle, APC/C^{Cdh1} drives cell differentiation through the destruction of two proteins, Skp2 and Myf5 (Li et al., 2007; **Figure 1**). Elimination of Skp2 leads to the accumulation of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ in myoblasts, allowing cell cycle withdrawal. Consistent with a role of $p21^{Cip1}$ in myotube formation, mice lacking $p21^{Cip1}$ fail to form myotubes (Zhang et al., 1999). Thus, the APC/C–Skp2–$p21^{Cip1}$/$p27^{Kip1}$ axis is likely to be essential for muscle development.

Coupled to APC/C mediated destruction of Skp2 in muscle is APC/C targeting of Myf5. Myf5 is a bHLH transcription factor that regulates myoblast proliferation and homeostasis (Gayraud-Morel et al., 2007). Its expression is restricted to dividing and undifferentiated cells. Myf5 is not directly involved in the decision to differentiate *per se* but in *Myf5* null animals, differentiation is delayed during early regeneration and *Myf5* null mutants are characterized by a subtle progressive myopathy and muscle regeneration deficits (Gayraud-Morel et al., 2007). Degradation of Myf5 by APC/C^{Cdh1} facilitates myogenic fusion, a process required for myoblast differentiation (Gayraud-Morel et al., 2007). Importantly, impaired degradation of both Skp2 and Myf5 seems to have a role in muscle cancer since overexpression of Skp2 and Myf5 are found in cervical carcinoma and rhabdomyosarcomas, respectively (Tamamori-Adachi et al., 2004; Zibat et al., 2010).

Lens cell differentiation

Proper lens differentiation requires precise temporal control of the cell cycle and the coordination of cell cycle exit with differentiation cues and signaling pathways (Zhu and Skoultchi, 2001). APC/C^{Cdh1} has been identified as a crucial regulator of lens differentiation that induces SnoN degradation (Wu et al., 2007). SnoN, a critical transcriptional corepressor of the TGF-β pathway, has been proposed to be a functional switch controlling the expression of p15 and $p21^{Cip1}$ (Zhu et al., 2005). $p21^{Cip1}$ and p15 are two essential cell cycle inhibitors that contribute to cell cycle arrest via downregulation of cyclin D/CDK4/6 activity (Reynisdottir et al., 1995). The upregulation of the $p21^{Cip1}$ and p15 inhibitors is necessary for coupling cell cycle withdrawal in response to TGF-β signaling and the initiation of lens differentiation. Moreover, *Cdh1* depletion was shown to attenuate induction of p15 and $p21^{Cip1}$ and significantly block lens differentiation (Wu et al., 2007). Impaired regulation of SnoN has not been related to corneal tumors but given the importance of APC/C^{Cdh1} in lens differentiation, further studies could open new potential targets in these carcinomas.

Hematopoietic cell differentiation

Hematopoiesis gives rise to all blood cells through a complex series of proliferation and differentiation events that occur throughout lifespan (Kawamoto et al., 2010). The hematopoietic system consists of a large array of differentiated blood cells including erythrocytes and cells of the myeloid and lymphoid lineages. Evidence supporting a role for APC/C^{Cdh1} in hematopoiesis comes from Cdh1 knockout mice. Cdh1 heterozygous mice develop B-cell lymphoma and myelodysplastic disorder (Garcia-Higuera et al., 2008). Although how APC/C^{Cdh1} regulates hematopoiesis remains to be investigated, targeted degradation of Id protein could be one mechanism (**Figure 1**). While Id proteins are downregulated during cell cycle exit, overexpression of Id proteins in terminally differentiated cells triggers cell cycle re-entry. Id proteins modulate cellular proliferation and differentiation in hematopoietic cells (Perk et al., 2005). Id1 is essential for hematopoietic stem cell maintenance and hematopoietic development (Perry et al., 2007) and the balance between Id1 and E-protein regulates myeloid-versus-lymphoid lineage commitment (Cochrane et al., 2009). Dysfunction of *Id2* in mice or cultured cells induces lymphoid differentiation, whereas Id2 overexpression inhibits lymphoid and myeloid differentiation (Perk et al., 2005; Ji et al., 2008). Furthermore, in addition to an Id1 and Id2 requirement for the proliferation and differentiation of hematopoietic precursors, these factors may contribute to the development of myeloid malignancies through enhanced proliferation or inhibited differentiation (Perk et al., 2005).

APC/C^{Cdh1} PRESERVES CHROMOSOME INTEGRITY

Loss of *Cdh1* function produces precocious initiation of DNA synthesis, leading to lower S phase progression, which results in stalled replication forks and under-replicated DNA (Garcia-Higuera et al., 2008). These replicative defects, as well as the upregulation of mitotic kinases, can ultimately lead to genetic damage (Garcia-Higuera et al., 2008; Cotto-Rios et al., 2011). *Cdh1*-deficient cells exhibit defects in mitotic exit and cytokinesis and accumulate a variety of genomic aberrations (Engelbert et al., 2008; Garcia-Higuera et al., 2008). Such genomic aberrations may be a consequence of the inability of APC/C^{Cdh1} to properly mediate a response to DNA damage in *Cdh1* null cells. In G1, APC/C^{Cdh1} targets the ubiquitin specific protease USP1 for proteasome dependent degradation (Cotto-Rios et al., 2011). Since USP1 counteracts monoubiquitination of the DNA repair protein proliferating cell nuclear antigen (PCNA), APC/C^{Cdh1} dependent USP1 destruction allows a permissive environment during G1 for PCNA monoubiquitination, which is required for UV-mediated DNA gap repair (Cotto-Rios et al., 2011). In addition to USP1, APC/C^{Cdh1} controls the levels of multiple proteins involved in the DNA damage checkpoint response and DNA repair including Claspin, Rad17, thymidine kinase 1, and the ribonucleotide reductase subunit (Chabes et al., 2003; Ke et al., 2005; Bassermann et al., 2008; Gao et al., 2009a; Zhang et al., 2010). During G2, the APC/C^{Cdh1} substrate polo-kinase 1 (Plk1) controls CDK1 activation and recovery from DNA replication stresses (Watanabe et al.,

2004; Mamely et al., 2006). Cells exposed to genotoxic stress in G2 need to arrest the cell cycle and repair damaged DNA. One mechanism to achieve this is to reduce levels of proteins such as Plk1 and mitotic cyclins, which are required for mitotic entry. Although normally inactive during G2, APC/C^{Cdh1} is activated via Cdh1 dephosphorylation during genotoxic stress, which is mediated by the Cdc14B phosphatase (Bassermann et al., 2008). Therefore, participation of APC/C^{Cdh1} in the G2 DNA damage response suggests another mechanism for genomic instability observed in *Cdh1* null cells.

APC/C^{Cdh1} MODULATORS AS TUMOR SUPPRESSOR PROTEINS OR ONCOGENES

Considering the prominent role of APC/C in cell cycle regulation and genomic stability, it is tempting to speculate that its dysregulation triggers a major perturbation of cell cycle progression and contributes to cell transformation. In fact, hemizygous frameshift and point mutations in several subunits of APC/C have been found in colon cancer cell lines and tumors (Wang et al., 2003). Mutations in *Apc3*, *Apc6*, and *Apc8* were found in colon cancer, breast cancer, neuroblastoma, hepatocarcinoma, melanoma, glioma, choriocarcinoma, endometrial cancer, ovarian cancer, and prostate carcinoma (Wang et al., 2003). Overexpression studies of Apc8 suggested that APC/C mutations can act in a dominant-negative manner to inhibit its function and cause inappropriate cell cycle progression (Wang et al., 2003). Moreover, the APC/C cofactor *Cdh1* has been described as a *tumor* suppressor (Garcia-Higuera et al., 2008). Knockdown or inhibition of *Cdh1* is associated with centrosome amplification and chromosome mis-segregation, and is implicated in genomic instability and tumorigenesis (Ross and Cohen-Fix, 2003; Wasch and Engelbert, 2005; Engelbert et al., 2008; Garcia-Higuera et al., 2008). *Cdh1*-deficient mice display genomic instability and heterozygous *Cdh1* mice develop epithelial tumors, such as mammary gland adenocarcinomas and fibroadenomas, which are not observed in wild-type mice. Furthermore, these tumors contain one *Cdh1* wild-type allele, suggesting that *Cdh1* is haploinsufficient for tumor suppression. In addition to mutations in APC/C or Cdh1 subunits, it is tempting to speculate that alterations of APC/C upstream regulators may lead to transformation (**Table 1**).

RETINOBLASTOMA PROTEIN

A well-established tumor suppressor protein that positively regulates APC/C activity is the pRB. pRB plays multiple roles during the cell cycle; it blocks cell cycle progression from G1 to S phase, and guards the cell from replicating damaged DNA, thus preventing the integration of mutations into the genome. This tumor suppressor function requires binding and repressing the E2F family transcription factors (Classon and Harlow, 2002; Chen et al., 2009). Reversion of the pRb–E2F interaction occurs after mitogenic stimuli sufficient for the activation of CDKs that phosphorylate pRB in the nucleus. Subsequently, phospho-pRb is released from E2Fs, which can then induce S phase entry (Thoma et al., 2011). Consistent with a direct inhibitory role in S phase entry, loss of various pRBs leads to unscheduled cell proliferation in many tissues (Vidal and Koff, 2000; Cobrinik, 2005; Marx et al., 2010). Accordingly, mutations in *Rb* are found in a wide variety of cancers mainly in the lung, breast, and eye (Meraldi et al., 1999; Sabado Alvarez, 2008).

Retinoblastoma protein also possesses E2F-independent functions that contribute to cell cycle control. pRB interacts with both Skp2 (Ji et al., 2004) and with APC/C^{Cdh1} through distinct surfaces (Binné et al., 2007). pRB needs to interact with both proteins to target Skp2 for ubiquitin-mediated degradation and promote p27^{Kip1} accumulation and cell cycle exit. Interestingly, similar to E2F1, Cdh1 only interacts with the hypophosphorylated (active) form of pRb, and phosphorylation of pRb by cyclin A/CDK2 kinase abolished the ability of Rb to interact with both Cdh1 and E2F1. Therefore Cdh1, by competing with pRb interaction, can also regulate the activity of the E2F1 transcription factor (Sorensen et al., 2000; Gao et al., 2009a). Further, given that both pRB and Cdh1 have tumor suppressive functions, whether the pRB–APC/C^{Cdh1} interaction is misregulated during tumorigenesis should be investigated (**Figure 2**).

PTEN

Recent studies have demonstrated that the well-characterized tumor suppressor protein PTEN is a positive regulator of APC/C activity. PTEN (Cairns et al., 1997; Steck et al., 1997; Feilotter et al., 1998; Gray et al., 1998; Li and Sun, 1998) encodes a lipid phosphatase, which plays a crucial role in adhesion, migration, growth, and apoptosis. *Pten*-null mice die embryonically, but heterozygous mice survive and develop tumors in the lymphoid system, endometrium, prostate, and the thyroid (Di Cristofano et al., 1998; Podsypanina et al., 1999). *Pten* somatic mutations occur in a large percentage of human cancers, with the highest numbers found in endometrial, central nervous system, skin, and prostate cancers (Chalhoub and Baker, 2009).

The effects observed after PTEN loss have been attributed to activation of the PI3K/AKT pathway as PTEN can dephosphorylate phosphoinositide-3,4,5-triphosphate (PIP3), a potent activator of Akt1 (Maehama and Dixon, 1998). PTEN inactivation by phosphorylation or oxidation in human cancer results in elevated Akt1 activity and abnormal growth regulation (Silva et al., 2008; Chalhoub and Baker, 2009; McCubrey et al., 2011). However, functions independent of its phosphatase activity have been described (Maier et al., 1999; Georgescu et al., 2000; Koul et al., 2002a,b; Gildea et al., 2004; Blanco-Aparicio et al., 2007). Recently, a novel interaction between PTEN and APC/C^{Cdh1} has been reported (Song et al., 2011). Song et al. (2011) demonstrated that nuclear PTEN directly enhances the activity of APC/C by promoting its association with Cdh1 in a phosphatase-independent manner. Conversely, PTEN loss impairs the activity of the APC/C^{Cdh1} complex. Therefore, loss of PTEN function decreases APC/C^{Cdh1} activity, suggesting novel molecular pathways that may be involved in the progression and initiation of tumorigenesis. This in turn highlights the complexity of the dose-dependent tumor promoting and fail-safe cellular responses evoked by PTEN loss. Accordingly, tumor-derived mutations that do not affect the phosphatase enzymatic activity of PTEN *in vitro* have also been identified. These were found to affect the compartmentalization of PTEN in the cell, either by sequestering PTEN in the nucleus (Denning et al., 2007) or by interfering with PTEN's recruitment to the plasma membrane (Lee et al., 1999; Georgescu et al., 2000; Walker et al.,

Table 1 | Modulators of APC/C^{Cdh1} dysregulated in cancer.

APC/C regulator	Effects on APC/C	Major cell cycle function (s)	Role in tumorigenesis	Relevant cancers	Relevant reference
PTEN	Activator. Promotes the interaction between APC/C and Cdh1	Promotes cell differentiation. Downregulation promotes cell proliferation	Acts as a tumor suppressor. Null mice die and heterozygous develop tumors	Lymphoid system, endometrium, thyroids, central nervous system, skin, prostate, breast	Chalhoub et al. (2009), Hollander et al. (2011), Song et al. (2011), Uddin et al. (2004), Kwon et al. (2008)
pRB	Activator. Promotes degradation of Skp2 by interaction with APC/C and Skp2	Blocks progression of G1/S. Downregulation promotes cell proliferation	Acts as a tumor suppressor. Prevents integration of DNA damage	Lung, breast, eye	Chen et al. (2009), Classon and Harlow (2002), Ji et al. (2004), Binné et al. (2007), Sabado Alvarez (2008)
Sirt2	Activator. Acetylates Cdh1 and Cdc20 promoting their interaction with APC/C	Regulates chromosomal condensation during mitosis. Maintains genome integrity	Acts as a tumor suppressor. Decrease of function promotes genome instability	Breast, liver, brain, kidney, and prostate cancers	North and Verdin (2007), Kim et al. (2011)
PSMA	Activator. Associates with Cdc27	Unknown	Acts as an oncogene. Overexpression promotes premature activation of APC/C and aneuploidy	Prostate cancer	Rajasekaran et al. (2005, 2008), Burger et al. (2002), Chang et al. (1999, 2001)
Emi1	Inhibitor. Binds to APC/C	Permits accumulation of cyclins in G1/S	Overexpression with p53 knock down promotes proliferation and chromosomal instability	Kidney, liver, lung, endometrium, lymphoid system, ovarium, lung	Hsu et al. (2002), Miller et al. (2006), Lehman et al. (2006), Gütgemann et al. (2008)
MAK	Inhibitor. Phosphorylates Cdh1	Promotes stabilization of APC/C^{Cdh1} substrates	Acts as an oncogene. Overexpression promotes extra-centrosomes	Prostate cancer	Wang and Kung (2011)

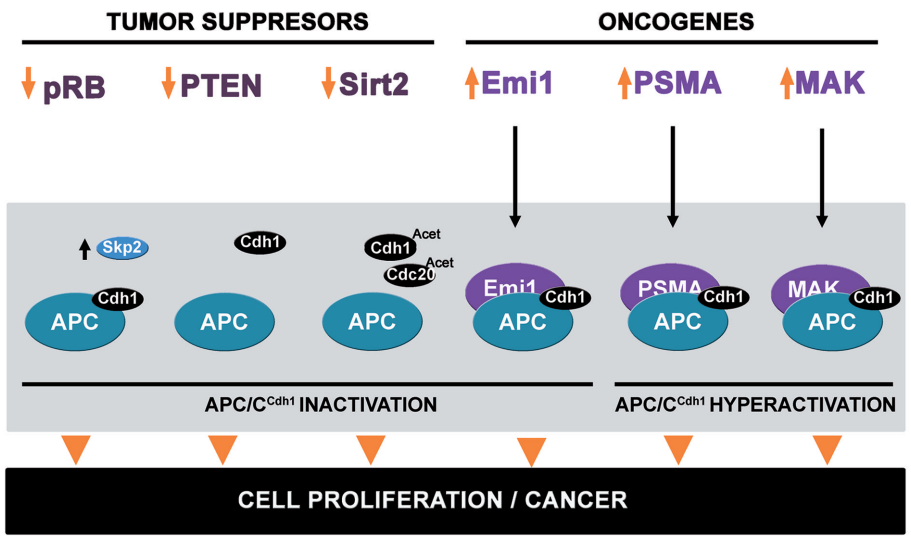

FIGURE 2 | Dysregulation of APC/C^{Cdh1} modulators can promote cancer. Decreasing expression of the tumor suppressors pRB, PTEN, and Sirt2 may promote APC/C^{Cdh1} inactivation, leading to cell proliferation and tumorigenesis. pRB downregulation may decrease Skp2 destruction. Decreasing PTEN levels might reduce APC/C activity. Decrease of Sirt2 could enhance the acetylation of the coactivators Cdh1 and Cdc20, lowering the capacity to interact with APC/C. In addition to the tumor suppressors, the oncogenes Emi1, PSMA, and MAK also regulate APC/C^{Cdh1} activity. Emi1 binds as a pseudo-substrate and leads to APC/C inactivation. PSMA and MAK increase APC/C^{Cdh1} activity and produce genomic instability.

2004), thus possibly negatively impacting the PTEN–APC/C^{Cdh1} interaction. Consistent with this possibility, APC/C^{Cdh1} substrates are overexpressed in tumors containing PTEN deficiency (Marino et al., 2002; Gao et al., 2009a; Liu et al., 2011b). Further, PTEN overexpression has been found to suppress tumorigenesis by inducing G1 cell cycle arrest in human glioblastoma cells (Li and Sun, 1998). Given the importance of PTEN and APC/C^{Cdh1} for cancer progression, it will be crucial to determine whether tumors containing PTEN misregulation have both hyeractivation of the Akt1 pathway and dysregulation of APC/C^{Cdh1} function (**Figure 2**).

EARLY MITOTIC INHIBITOR-1

The Emi1 is an APC/C inhibitor, which is overexpressed in multiple tumors. Emi1 is a key cell cycle regulator that is required for accumulation of mitotic cyclins and other critical cell cycle regulators during S phase and G2 (Hsu et al., 2002). At the G1/S transition, Emi1 functions as a pseudo-substrate inhibitor of the APC/C (Miller et al., 2006), allowing substrates to accumulate (Guardavaccaro et al., 2003; Miller et al., 2006). In early mitosis, Emi1 is phosphorylated by Plk1 (Hansen et al., 2004), which triggers $SCF^{\beta TrCP}$-dependent ubiquitination and destruction, thus inducing APC/C activation and mitotic progression (Margottin-Goguet et al., 2003). Emi1 overexpression leads to unscheduled cell proliferation, tetraploidy, and chromosomal instability in *p53*-deficient cells (Lehman et al., 2006; **Figure 2**). In *p53* wild-type cells, the induction of tetraploidy and aneuploidy by overexpressing APC/C inhibitors like Emi1 typically leads to G1 arrest or apoptosis. Indeed, Emi1 overexpression causes mitotic catastrophe and genomic instability through APC/C misregulation, and thus potentially contributes to tumorigenesis (Hsu et al., 2002). Upregulation of *Emi1* mRNA has been found in a variety of malignant tumors compared to matched normal and benign tumor tissue. Notably, Emi1 protein is highly expressed in renal cell carcinomas, cervical adenocarcinomas, hepatocellular carcinomas, oligodendrogliomas, lung adenocarcinomas, endometrial cancers, of melanomas, many lymphomas and ovarian clear cell carcinoma (Lehman et al., 2006; Gütgemann et al., 2008). Emi1 expression is also related to the pRB/E2F pathway since Emi1 is a target of the E2F transcription factor. At the G1-S transition Emi1 is transcriptionally induced by E2F, thus accelerating S phase entry (Hsu et al., 2002). The importance of this regulation is underscored by the finding that lack of pRb repression of E2F-mediated transcription causes misregulation of Emi1 and APC/C substrates in malignant tumors (Lehman et al., 2006).

Sirt2

Recently, the histone deacetylase (HDAC) SIRT2 has been shown to positively regulate APC/C activity (Kim et al., 2011). Histone acetyltransferases (HATs) and HDACs are enzymes controlling protein acetylation. Both target histones, whereas HATs catalyze the acetylation of histones and relax chromatin to increase accessibility of transcription factors to promoters of target genes, HDACs remove acetyl groups from histones and repress transcription (Strahl and Allis, 2000). Most of them have been found in transcription factor complexes and, therefore, have been considered to regulate transcription by modulating acetylation levels of chromatin (Kuo and Allis, 1998; Kouzarides, 1999). HDACs are important for cell cycle progression and their inhibition promote cell arrest in G1 and G2 (Marks et al., 2001; Johnstone and Licht, 2003; Noh and Lee, 2003). HDACs also have an important role in heterochromatin formation and maintenance (Olsson et al., 1999; Taddei et al., 2001) and are implicated in chromosome segregation (Nakayama et al., 2003; Silverstein et al., 2003; Kimata et al., 2008).

Several HDACs have a role in tumorigenesis, acting as a suppressors or promoters (Lagger et al., 2002; Glaser et al., 2003; Saunders and Verdin, 2007; Wang et al., 2008a,b; Deng, 2009; Bell et al., 2011; Kim et al., 2011) and HDAC inhibitors are a promising class of anti-cancer agents (Johnstone and Licht, 2003; Yoshida et al., 2003; Bolden et al., 2006). SIRT2 is predominantly localized in the cytoplasm where it deacetylates microtubules (North et al., 2003). During mitosis, SIRT2 is localized to chromosomes regulating chromosomal condensation (Vaquero et al., 2006; Inoue et al., 2007), and is also associated with mitotic structures, including the centrosome, mitotic spindle, and midbody, presumably to ensure normal cell division (North and Verdin, 2007). Kim et al. (2011) demonstrated that SIRT2 regulates APC/C activity through deacetylation of its coactivators Cdh1 and Cdc20. Deacetylation of Cdh1 and Cdc20 by SIRT2 enhances the interaction of these coactivators with Cdc27, leading to activation of APC/C. In *Sirt2* mutant mice, reduced APC/C activity results in tumor formation by producing genomic instability associated with centrosome amplification, aneuploidy mitotic cell death, and spontaneous tumor formation. These deficiencies seem to be caused by a combined effect of altered expression of mitotic regulators that are controlled by APC/C (**Figure 2**). Accordingly, SIRT2 expression is reduced in several human malignancies including breast, liver, brain, kidney, and prostate cancers (Kim et al., 2011).

PSMA AND MAK

Mitotic defects associated with chromosomal aberrations are often observed in prostate cancer cells and tissue, suggesting possible misregulation by APC/C (Tribukait, 1991; Beheshti et al., 2001; Pihan et al., 2001). Two regulators of the APC/C have been found to be clearly involved in prostate cancer, prostate specific membrane antigen (PMSA) and male germ cell-associated kinase (MAK). PSMA is a type II transmembrane protein that exhibits both *N*-acetylated alpha-linked acidic peptidase (NAALADase) and folate hydrolase activities (Ghosh and Heston, 2004; Rajasekaran et al., 2005). PSMA is localized to secretory cells within the prostatic epithelium although its physiological and pathological functions remain unclear. PSMA is upregulated in advanced prostate carcinoma and metastatic disease (Rajasekaran et al., 2005), and is a feature of practically every prostatic tissue examined (Rajasekaran et al., 2005, 2008). PSMA is absent or moderately expressed in hyperplastic and benign tissues, while malignant tissues have high levels, demonstrating that PSMA expression increases proportionally to tumor aggressiveness (Troyer et al., 1995; Kawakami and Nakayama, 1997; Liu et al., 1997; Silver et al., 1997; Sweat et al., 1998; Chang et al., 1999, 2001; Burger et al., 2002; Ross et al., 2003). PSMA is localized to a membrane compartment in the vicinity of centrosomes at the spindle poles and associates with the APC/C subunit Cdc27 leading to

premature activation of APC/C, and induction of aneuploidy (Rajasekaran et al., 2008). Increased APC/C activity observed in PSMA expressing cells is sufficient to impair the mitotic spindle checkpoint, which agrees with the finding that PSMA expressing cells exit mitosis prematurely (**Figure 2**). Therefore, PSMA may have a causal role in the progression of prostate cancer.

Male germ cell-associated kinase belongs to a protein kinase family characterized by a catalytic domain resembling a hybrid of the TXY motif found in mitogen-activated protein kinases (MAPK) and the TY motif in CDKs (Payne et al., 1991; Brown et al., 1999; Xia et al., 2002; Fu et al., 2005). MAK expression is elevated in castration-resistant prostate cancer cell lines and is generally overexpressed in prostate tumors, thus possibly contributing to malignancy via aberrant regulation of mitosis (Wang and Kung, 2011). MAK protein has a dynamic subcellular localization during the cell cycle: it is localized to the mitotic spindle and centrosomes during metaphase and anaphase, and to the mitotic midbody from anaphase to telophase. MAK negatively regulates APC/C^{Cdh1} through interaction and phosphorylation of Cdh1 in CDK phosphorylated sites, in a manner reminiscent of CDK-dependent inactivation of Cdh1 (Wang and Kung, 2011; **Figure 2**). The phosphorylation of MAK increases between S and G2, peaks at early mitosis, and drastically decreases at the end of mitosis. This promotes the dissociation of Cdh1 and APC/C thus decreasing APC/C^{Cdh1} activity and promoting the stabilization of the substrates Aurora kinase A and Plk1. As overexpression of Aurora-A is known to induce centrosome amplification (Zhou et al., 1998), the extra-centrosomes observed in MAK overexpressed cells is likely due to cellular accumulation of Aurora-A.

APC/C SUBSTRATES IN CANCER

Many APC/C substrates have been implicated in a variety of human cancers (**Table 2**). For those substrates that are overexpressed in cancer it is important to note that overexpression of an APC/C substrate influences degradation of other APC/C substrates (Rape and Kirschner, 2004), suggesting that global estimates of APC/C substrate levels are required in normal and disease states. Further, while there are arguably many APC/C substrates that may be important for cancer progression, classifying their relative importance and druggability is essential.

NIMA-RELATED KINASE 2

The proteolysis of APC/C substrates starts as cells make the transition from G2 to M phase. One of the earliest targets is the NIMA-related kinase 2 (Nek2) family of serine/threonine protein kinases. They are implicated in the regulation of centrosome separation and spindle formation. Nek2A, along with cyclin A are unique APC/C^{Cdc20} substrates that get degraded during prometaphase even when the SAC is active. A C-terminal dipeptide methionine–arginine (MR) tail enables Nek2A to directly bind the APC/C independently of Cdc20 (Hayes et al., 2006). Nek2 levels are upregulated in human breast cancer, pediatric osteosarcoma, and B-cell lymphomas (Wai et al., 2002; de Vos et al., 2003; Hayward et al., 2004).

CELL DIVISION CYCLE PROTEIN 20

Cell division cycle protein 20 is an essential coactivator of the APC/C during mitosis. Multiple studies have indicated that maintaining appropriate Cdc20 levels is important for cellular homeostasis. When this is disturbed and Cdc20 is overexpressed, tumorigenesis can occur. For instance, oral squamous cell carcinomas (OSCC) and breast cancer cells overexpress Cdc20 causing premature anaphase and aneuploidy linked to tumor formation (Yuan et al., 2006; Mondal et al., 2007). Importantly, even a twofold overexpression of Cdc20 leads to spindle checkpoint defects and early Pds1 (securin) destruction producing aneuploidy (Pan and Chen, 2004).

One of the major ways Cdc20 protein levels are controlled is via the SAC. When the SAC is active during metaphase, the checkpoint proteins Mad2, BubR1, and Bub3 bind to Cdc20 and convert it into a substrate of APC/C^{Cdc20}. This allows for the ubiquitination and degradation of Cdc20 by APC/C^{Cdc20} (Nilsson et al., 2008; Ge et al., 2009). Thus, SAC activation inhibits Cdc20 and arrests cells in metaphase. Inactivation of SAC at the end of metaphase relieves this inhibition and Cdc20 promotes mitotic exit by degrading key substrates such as the mitotic cyclins. The importance of SAC dependent control of Cdc20 levels is underscored by the finding that anti-mitotic cancer drugs that activate the SAC cause cell cycle arrest. These drugs cause a prolonged metaphase arrest that may eventually lead to cell death. However, during this prolonged metaphase arrest, some Cdc20 escapes inhibition by the SAC and as a result, cyclin B1 levels start to fall and cells exit mitosis, thereby initiating mitotic slippage (Nilsson, 2011). This may be especially true in some cancer subtypes since the frequency of mitotic slippage is hastened in tumor cells lacking p53 and pRb (Depamphilis, 2011). Further, mitotic slippage reduces the sensitivity of tumor cells to chemotherapeutics. Thus, alternative means of inhibiting Cdc20 are needed.

Recent evidence suggests that targeting Cdc20 directly could be a better alternative than targeting the SAC in cancer therapy (Huang et al., 2009; Manchado et al., 2010). Knockdown of Cdc20 by siRNA induces mitotic arrest and apoptosis in various cancer cell lines that are otherwise resistant to apoptosis and prone to mitotic slippage (Huang et al., 2009). Further, genetic ablation of the *Cdc20* gene in murine skin tumor and aggressive fibrosarcoma models results in complete tumor regression (Manchado et al., 2010). *Cdc20* deletion induces metaphase arrest and apoptosis in tumor cells both *in vitro* and *in vivo*. Importantly, the activities of Cdk1 and Mast1 kinases are required for this arrest. When Cdk1 and Mast1 kinases are inhibited, *Cdc20* null cells exit mitosis via the activities of the phosphatases PP2A/B55α and PP2A/B55δ (Manchado et al., 2010). Thus, small molecules attenuating Cdc20 coupled with those inhibiting these phosphatases may be effective therapeutically.

GEMININ

Another APC/C substrate degraded at the metaphase–anaphase transition is geminin. It is a small 25 kDa protein that inhibits DNA re-replication during S phase. Geminin levels accumulate during S, G2, and early mitosis. It suppresses the DNA replication factor CDT1 that is required for the formation of preRCs. Depletion of geminin by siRNA in tumor cells leads to DNA re-replication, cell

cycle arrest, and apoptosis. However, regulation of CDT1 by geminin is found to be rate limiting for initiation of DNA replication only in cancer cells and not in normal or immortalized cells. Normal cells show the same effect only when cyclin A is co-depleted with geminin (Zhu and Depamphilis, 2009). Thus it has been proposed that inhibition of geminin activity can be used to selectively kill cancer cells (Zhu and Depamphilis, 2009).

POLO-LIKE KINASE 1

Toward the end of anaphase, the pro-mitotic regulatory kinase Plk1, is degraded by APC/C^{Cdh1} in a D-box dependent manner (Lindon and Pines, 2004). Plk1 is a major kinase in eukaryotic cells involved in a variety of processes such as activation of the maturation promoting factor (MPF) by phosphorylation of Cdc25C and cyclin B1, bipolar spindle formation, and maturation of the centrosome (Ohkura et al., 1995; Lane and Nigg, 1996; Abrieu et al., 1998; Qian et al., 1998; Eckerdt et al., 2005). Elevated Plk1 levels are present in a broad spectrum of cancers including breast cancer, colorectal cancer, ovarian cancer, melanomas, pancreatic cancer, prostate cancer, lung cancer, and squamous cell carcinomas of the head and neck (Wolf et al., 1997, 2000; Knecht et al., 1999; Strebhardt et al., 2000; Macmillan et al., 2001; Gray et al., 2004; Weichert et al., 2004). This broad range of expression makes Plk1 a prime target in cancer (Strebhardt and Ullrich, 2006).

AURORA-A

Another APC/C^{Cdh1} substrate during late mitosis is Aurora-A (STK15/BTAK), a serine/threonine kinase localized to the centrosome controlling mitotic spindle assembly and centrosome maturation. It is involved in a variety of human cancers (Bischoff et al., 1998; Zhou et al., 1998; Sen et al., 2002; Mondal et al., 2007). Ectopic expression of Aurora-A leads to chromosome instability and centrosome amplification (Miyoshi et al., 2001). One way it achieves this is by phosphorylating and regulating Centrin, the calcium-binding phosphoprotein located in the centrosome. Phosphorylated Centrin is more stable against APC/C^{Cdh1} mediated destruction and an excess of it is thought to promote centrosome amplification in cancer (Lukasiewicz et al., 2011). Aurora-A itself becomes resistant to APC/C^{Cdh1} mediated degradation when it is constitutively phosphorylated on Ser 51, and consequently gets overexpressed in head and neck cancers (Kitajima et al., 2007).

HIGH EXPRESSION IN CANCER 1

High expression in cancer 1 (HEC1), a recently identified APC/C^{Cdh1} substrate, is part of the NDC80 complex controlling kinetochore microtubule dynamics. HEC1 levels peak during early mitosis and fall by telophase (Lipkowitz and Weissman, 2011). The mitotic regulatory kinase Nek2 phosphorylates HEC1 on Ser 165 thus activating it during G2/M. Inactivation of HEC1 results in severe chromosome segregation defects (Chen et al., 1997, 2002; Zheng et al., 1999). HEC1 is considered as a candidate marker for breast lesions likely to undergo malignant transformation because it is significantly overexpressed in benign breast tumors (Bieche et al., 2011). A small molecule called INH1, which binds to HEC1 and specifically disrupts the HEC1–Nek2 interaction, is found to suppress human breast cancer cell proliferation in culture as well as tumor growth in nude mice bearing xenografts (Wu et al., 2008).

c-Jun NH2-TERMINAL KINASE

c-Jun NH_2-terminal kinase (JNKs), a class of MAPK, lead the cell's response to stress stimuli such as heat shock, radiation, and cytotoxic and genotoxic stress. Recently, nuclear JNK was found to be a substrate of APC/C^{Cdh1} during late mitosis and G1 (Gutierrez et al., 2010a). JNK, in turn, controls APC/C^{Cdh1} by phosphorylating Cdh1 at three residues (Yu et al., 1998; Summers et al., 2008) to prevent premature Cdh1 association with the APC/C during G2. JNK-induced phosphorylation of key regulators not only controls cell survival and differentiation, but also cell cycle progression. JNK phosphorylates Cdc25C at Ser 168 in G2, downregulating its phosphatase activity required for CDK1 activation (Gutierrez et al., 2010b). This is required for the correct timing of mitotic entry and the proper establishment of G2/M checkpoint upon UV irradiation. However, constitutively active JNK causes defects in cell cycle progression. Indeed, many human tumors have been reported to require JNK activity for their growth and survival (Potapova et al., 2000; Antonyak et al., 2002; Tsuiki et al., 2003; Yang et al., 2003; Lopez-Bergami et al., 2007; Alexaki et al., 2008). In gastrointestinal cancers, a small molecule inhibitor of JNK shows promise as a therapeutic agent, because it induces cell cycle arrest and apoptosis (Xia et al., 2006).

Ect2

The oncogenic protein Ect2 is ubiquitinated by APC/C^{Cdh1} shortly after completion of mitosis (Liot et al., 2011). Ect2 is a positive regulator of the Rho GTPase pathway that controls cell cycle progression and cytokinesis (Bustelo et al., 2007). Many human tumors overexpress Ect2 (Sano et al., 2006; Salhia et al., 2008; Justilien and Fields, 2009; Justilien et al., 2011). Until now, this overexpression was believed to be a result of gene amplification and transcriptional upregulation (Seguin et al., 2009; Liot et al., 2011). But the newly discovered APC/C^{Cdh1} dependent proteolysis of Ect2 shows that impaired destruction can also be a reason for increased Ect2 levels in tumor cells, which possibly asserts the role of Cdh1 as a tumor suppressor protein.

Skp2

Skp2 is one of the most important substrates targeted by APC/C^{Cdh1} during mitotic exit. An SCF containing Skp2 targets the essential cell cycle substrates $p27^{Kip1}$, $p21^{Cip1}$, and FOXO1 for degradation (Yu et al., 1998; Tsvetkov et al., 1999; Huang et al., 2005). Destruction of the CDK inhibitor $p27^{Kip1}$ is a prerequisite for entry into mitosis. Once cells exit mitosis, APC/C^{Cdh1} targets Skp2 for proteolysis in G1 (Bashir et al., 2004; Wei et al., 2004). Skp2 functions as an oncogene because most of its ubiquitination targets are tumor suppressor proteins. Thus overexpression of Skp2 is common in many cancers including breast cancer, prostate cancer, pancreatic cancer, hepatocellular carcinomas, melanomas, and malignant lymphomas (Lim et al., 2002; Yang et al., 2002; Radke et al., 2005; Lu et al., 2009; Rose et al., 2011; Schuler et al., 2011). Importantly, the Akt1 serine/threonine kinase phosphorylates Skp2 at Ser 72 promoting its cytoplasmic translocation and impairing degradation by APC/C^{Cdh1} (Gao et al., 2009b,c; Lin et al., 2009). Since the Akt1 pathway is also found to be hyperactive in cancer cells, it may contribute significantly to overexpression of Skp2 protein found in some cancers (Gao et al.,

2009b,c). Opposing the Akt1 pathway is PTEN, which is frequently misregulated in human brain, breast, prostate cancers, and leukemias (Li et al., 1997; Dahia et al., 1999). Thus, PTEN misregulation may contribute to Akt1 hyperactivation and increased levels of Skp2 in tumors.

Ube2C (UbcH10)

Ube2C is an E2 enzyme that works exclusively with the APC/C throughout the cell cycle. However, Ube2C levels peak during mitosis and fall as cells enter G1, finally becoming a substrate for APC/C^{Cdh1} at the end of G1 (Rape and Kirschner, 2004; Summers et al., 2008). When Ube2C is overexpressed in mouse embryonic fibroblasts (MEFs), precocious cyclin B1 degradation, mitotic slippage, and chromosome abnormalities have been observed (van Ree et al., 2010). Importantly, elevated Ube2C levels is a common feature in a wide variety of human cancers including lung, prostate, breast, bladder, ovarian, uterine, thyroid, esophageal, and gastric carcinomas (Okamoto et al., 2003; Pallante et al., 2005; Berlingieri et al., 2007; Jiang et al., 2008; van Ree et al., 2010). Further, induced expression of high levels of Ube2C hastens tumor formation in transgenic mice (van Ree et al., 2010).

MODULATING APC/C^{Cdh1} ACTIVITY IN CANCER

TARGETING APC/C^{Cdh1} SUBSTRATES

Several APC/C substrates are overexpressed in various cancers, making them prime targets for small molecule inhibition (**Table 2**). One potential APC/C substrate that could be targeted

Table 2 | Substrates of APC/C overexpressed in cancer.

APC/C substrate	Major cell cycle function(s)	Role in tumorigenesis	Relevant cancers	Relevant reference
Nek2	Regulation of centrosome separation and spindle formation	Formation of multinucleated cells with supernumerary centrosomes	Cervical cancer, B-cell lymphoma, pediatric osteosarcoma, breast cancer, leukemia, ovarian cancer	Wai et al. (2002), de Vos et al. (2003), Hayward et al. (2004)
Cdc20	Essential APC/C coactivator in early mitosis	Spindle checkpoint defects and premature Securin destruction leading to mitotic slippage	Oral squamous cell carcinomas, breast cancer	Yuan et al. (2006), Mondal et al. (2007)
Plk1	Activation of MPF by phosphorylation of Cdc25C and Cyclin B Assembling the mitotic spindle	Overexpression in cancer drives cells into mitosis	Lung, ovarian, breast, colon, prostate and pancreatic cancers, head/neck squamous cell carcinomas, melanomas	Wolf et al. (1997), Knecht et al. (1999), Strebhardt et al. (2000), Wolf et al. (2000), Macmillan et al. (2001), Gray et al. (2004), Weichert et al. (2004)
Aurora-A	Controls centrosome maturation and mitotic spindle formation	Induces centrosome amplification and aneuploidy by hindering APC/C^{Cdh1} mediated destruction of Centrin	Bladder, lung, and colon cancers	Bischoff et al. (1998), Zhou et al. (1998), Sen et al. (2002), Gu et al. (2007)
HEC1	Controls kinetochore microtubule dynamics as part of the NDC80 complex	Chromosome mis-segregation	Breast cancer	Bieche et al. (2011)
JNK	Central role in stress response pathways Ensures correct timing of mitotic entry by phosphorylating Cdc25C	Supports survival of tumor cells by controlling cell cycle arrest and apoptosis	Brain tumors such as glioblastoma, prostate cancer, gastrointestinal cancers, melanomas	Potapova et al. (2000), Antonyak et al. (2002), Tsuiki et al. (2003), Yang et al. (2003), Xia et al. (2006), Lopez-Bergami et al. (2007), Alexaki et al. (2008)
Ect2	Positive regulator of the Rho GTPase pathway that controls actin cytoskeleton functions like cytokinesis	Ensures proper cytokinesis in tumor cells	Glioblastoma, non-small cell lung cancer	Salhia et al. (2008), Justilien et al. (2011)
Skp2	F-box protein that functions as part of SCF^{Skp2} to degrade Cdk inhibitors $p27^{Kip1}$ and $p21^{Cip1}$	Acts as an oncogene that destroys tumor suppressor proteins	Breast, prostate, liver and pancreatic cancers, melanomas, lymphomas	Lim et al. (2002), Radke et al. (2005), Lu et al. (2009), Rose et al. (2011), Schuler et al. (2011)
Ube2C (UbcH10)	Principal E2 enzyme the APC/C collaborates with	Chromosome abnormalities and mitotic slippage	Gastric carcinomas, cancers of the lung, prostate, breast, ovary, bladder, thyroid, uterus, and esophagus	Okamoto et al. (2003), Pallante et al. (2005), Berlingieri et al. (2007), Jiang et al. (2008), van Ree et al. (2010)

via such a strategy is the proto-oncogene Skp2 (Fujita et al., 2008, 2009). Downregulation of Skp2 by antisense RNA treatment induces apoptosis in lung cancer cells (Yokoi et al., 2003). Therefore, one possibility is utilizing compounds affecting Skp2 expression. For instance, treatment with EB1089 (vitamin D analog) reduces Skp2 expression in treated cancer cells, thus promoting increased stability of $p27^{Kip1}$ protein and subsequent growth arrest (Lin et al., 2003; **Figure 3**). Skp2 expression is also affected by treatment with retinoic acid, thus altering the ability of $p27^{Kip1}$ to be ubiquitinated (Nakamura et al., 2003). Furthermore, the PPARg agonist Troglitazone reduces *Skp2* mRNA levels, leading to $p27^{Kip1}$ accumulation (Koga et al., 2003).

Since the APC/C mediates Skp2 ubiquitination, enhancing APC/C dependent degradation of Skp2 in cancer would also be therapeutically attractive. One possible means of achieving this is to inhibit casein kinase 1 (CK1), which controls APC/C dependent degradation of Skp2 (Gao et al., 2009b,c). CK1 inhibition induces Skp2 degradation since CK1 dependent phosphorylation normally inhibits Skp2 nuclear translocation and interaction with APC/C^{Cdh1} (Gao et al., 2009b,c). An alternative strategy, however, could be increasing the affinity of Skp2 or other oncogenic substrates for APC/C, thus reducing their protein levels after ubiquitination and degradation. The advent of high-throughput screens to study APC/C-dependent degradation and protein–protein interactions will facilitate identification of small molecule agonists of the APC/C–Skp2 interaction (Madoux et al., 2010; Zeng et al., 2010).

Another potential APC/C cancer target is the substrate Aurora-A, since its overexpression is known to induce centrosome amplification and is overexpressed in human cancer cells (Zhou et al., 1998). Recently, several compounds have been found to target Aurora-A (**Figure 3**). Curcumin, an active compound in turmeric and curry that has been proven to induce tumor apoptosis and inhibit tumor proliferation, invasion, angiogenesis, and metastasis, has been shown to reduce *Aurora-A* mRNA expression in human bladder cancer cells (Liu et al., 2011a). These curcumin-induced phenomena were similar to those using Aurora-A small interfering RNA and were attenuated by ectopic expression of Aurora-A. Also, several cyclic peptide ligands inhibiting Aurora-A kinase activity have been developed (Shomin et al., 2011), although future studies regarding proliferation of cancer cells still have to be performed.

In addition to Aurora-A inhibition, there is pharmaceutical interest in targeting Plk1 as Plk1 depletion in cancer cells dramatically inhibits cell proliferation and decreases viability (Liu and Erikson, 2003; Xu et al., 2011; Yoon et al., 2011). Depletion of Plk1 perturbs spindle assembly, which leads to activation of the mitotic checkpoint, prolonged mitotic arrest, and eventually apoptosis (Liu and Erikson, 2003). Thus, given the known functions and effects of Plk1 inhibition, coupled with its wide overexpression pattern in cancer, a naturally occurring Plk1 inhibitor with low or no toxicity will be immensely useful in prevention as well as treatment of cancer. Numerous Plk1 inhibitors are in development to evaluate their potential as treatments in oncology, some of them in preclinical and clinical phase I/II development (Schöffski, 2009; **Figure 3**). It will be interesting to determine whether enhancing APC/C mediated degradation of Plk1 yield similar therapeutic benefits as Plk1 inhibitors currently in clinical trials.

TARGETING APC/C^{Cdh1} ACTIVITY

Several lines of evidence suggest that modulation of the APC/C complex is a good anti-cancer therapeutic strategy. Downregulation of the catalytic subunits Apc2 and Apc11 leads to growth arrest and cell death in HeLa cells (Pray et al., 2002). Also, a small molecule, tosyl-L-arginine methyl ester (TAME), which binds to APC/C and prevents its activation by Cdc20 and Cdh1, produces mitotic arrest (Zeng et al., 2010; **Figure 3**). Furthermore, expression of several APC/C subunits is higher in tumor relative to normal tissue (Wang et al., 2003). Collectively, these studies suggest that pharmacological interference with APC/C activity through disruption of protein–protein interaction is likely to have potent anti-tumor activity (Pray et al., 2002; Huang et al., 2009).

Targeting upstream regulators of the APC/C complex controlling mitosis is also a promising strategy. Overexpression of APC/C inhibitors such as Emi1 in mammalian cells impedes cell cycle progression and results in cell death (Reimann et al., 2001). HDACs

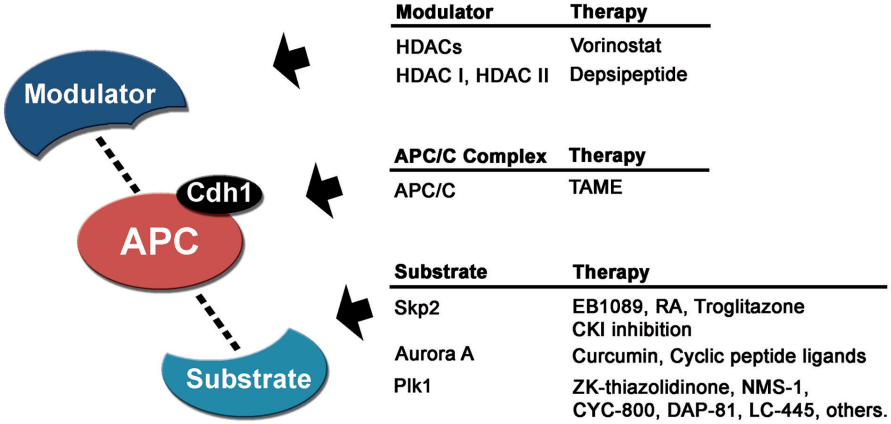

FIGURE 3 | Cancer therapeutics related to APC/C^{Cdh1}. Targeting the modulators, the APC/C^{Cdh1} complex or its substrates could be a good anti-cancer strategy. Some pharmacological treatments that regulate APC/C activity or substrate levels are currently in clinical trials or under development.

might directly target APC/C to ensure proper chromosome segregation and anti-tumor effects of HDAC inhibitors could be attributed to this deregulation (Kimata et al., 2008). There are emerging HDAC inhibitors that have been clinically validated as therapeutic agents in cancer patients with hematologic malignancies. Two HDAC inhibitors, vorinostat (pan-HDAC inhibitor) and depsipeptide (HDAC 1 and II inhibitor) have been approved by the FDA and are under further clinical investigation (**Figure 3**). HDAC inhibitors are well tolerated and clinically effective against hematologic cancers (Duvic and Vu, 2007; Luu et al., 2008; Modesitt et al., 2008).

CONCLUDING REMARKS

The APC/C is a unique ubiquitin ligase since it possesses a distinct structure and interacts with specific E2 enzymes required for mediating substrate ubiquitination. Further, APC/C^{Cdc20} activity is essential *in vitro* and *in vivo*, suggesting that no other ubiquitin ligase can substitute for APC/C's role during mitosis (Li et al., 2007; Manchado et al., 2010). Similarly, APC/C^{Cdh1} activity is required for cell cycle traverse, differentiation, placental formation, and for inhibiting tumorigenesis (Garcia-Higuera et al., 2008; Li et al., 2008). These exciting studies suggest that APC/C plays a large role in normal cell proliferation, which may be misregulated during cancer. Consistent with this notion are new studies linking APC/C substrates and regulators to tumor formation. Thus, it may be attractive to modulate APC/C substrate or regulator interaction pharmacologically to ablate tumor growth, which would have the advantage that specific interactions relevant to a cancer type can be uniquely targeted.

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The role of Elongin BC-containing ubiquitin ligases

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The Elongin complex was originally identified as a positive regulator of RNA polymerase II and is composed of a transcriptionally active subunit (A) and two regulatory subunits (B and C). The Elongin BC complex enhances the transcriptional activity of Elongin A. "Classical" SOCS box-containing proteins interact with the Elongin BC complex and have ubiquitin ligase activity. They also interact with the scaffold protein Cullin (Cul) and the RING domain protein Rbx and thereby are members of the Cullin RING ligase (CRL) superfamily. The Elongin BC complex acts as an adaptor connecting Cul and SOCS box proteins. Recently, it was demonstrated that classical SOCS box proteins can be further divided into two groups, Cul2- and Cul5-type proteins. The classical SOCS box-containing protein pVHL is now classified as a Cul2-type protein. The Elongin BC complex containing CRL family is now considered two distinct protein assemblies, which play an important role in regulating a variety of cellular processes such as tumorigenesis, signal transduction, cell motility, and differentiation.

Keywords: ubiquitin, Cullin, Elongin, ECS complex, SCF complex

INTRODUCTION

Polyubiquitin-mediated protein degradation plays an important role in the elimination of short-lived regulatory proteins (Peters, 1998), including those that contribute to the cell cycle, cellular signaling in response to environmental stress or extracellular ligands, morphogenesis, secretion, DNA repair, and organelle biogenesis (Hershko and Ciechanover, 1998). The system responsible for the attachment of ubiquitin to the target protein consists of several components that act in concert (Hershko and Ciechanover, 1992; Scheffner et al., 1995), including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin–protein isopeptide ligase (E3). E3 is believed to be the component of the ubiquitin conjugation system that is most directly responsible for substrate recognition (Scheffner et al., 1995). Based on structural similarity, E3 enzymes have been classified into three families: the HECT (homologous to E6-AP COOH terminus) family (Huibregtse et al., 1995; Hershko and Ciechanover, 1998), the RING finger-containing protein family (Lorick et al., 1999; Freemont, 2000; Joazeiro and Weissman, 2000), and the U box family (Aravind and Koonin, 2000; Hatakeyama et al., 2001; Cyr et al., 2002). The S phase kinase-associated protein 1 (Skp1)–Cullin 1 (Cul1)–F box protein (SCF) family is a member of the RING finger-containing ubiquitin ligase family (Lipkowitz and Weissman, 2011). Cul1 is a scaffold protein and assembles multiple proteins into complexes, which include a small RING finger protein (Rbx1), an adaptor protein (Skp1), and a substrate-targeting protein (F box protein). Substrate recognition by the RING finger-containing ubiquitin ligase family is modulated by post-translational modifications of the target substrate, including phosphorylation, glycosylation, and sumoylation (Lipkowitz and Weissman, 2011). One substrate can be polyubiquitinated by different ubiquitin ligases and *vice versa*. The Elongin B and C–Cul2 or Cul5–SOCS box protein (ECS) family also belongs to the Cullin RING ligase (CRL) superfamily (Kile et al., 2002). SCF and ECS ubiquitin ligases have structural similarities in that both contain Rbx1 or Rbx2 as a RING finger protein and Cul1, Cul2, or Cul5 as a scaffold protein (Kile et al., 2002; Kamura et al., 2004). Although Skp1 is used as an adaptor protein in the SCF complex, the Elongin B and C complex is used as an adaptor in the ECS complex. Here, we review the Cul2- or Cul5-containing ECS ubiquitin ligase family, about which, compared to SCF ubiquitin ligases, relatively little is known.

THE ELONGIN COMPLEX

The Elongin complex is a positive regulator of RNA polymerase II (pol II) and increases the rate of elongation by suppressing transient pausing along the DNA template (Bradsher et al., 1993a,b). The Elongin complex is composed of a transcriptionally active A subunit and two regulatory subunits, B and C (Garrett et al., 1994, 1995; Aso et al., 1995). Elongin B and C form the Elongin BC complex, which enhances the transcriptional activity of Elongin A. Since Elongin B and C partially resemble ubiquitin and Skp1 (an adaptor of SCF-type ubiquitin ligases), respectively (Bai et al., 1996), they are able to serve as components of protein complexes with functions other than transcriptional regulation. For example, they were found to be components of the von Hippel–Lindau (VHL) tumor suppressor complex, which is also known as the ECS complex (**Figure 1**; Duan et al., 1995; Kibel et al., 1995).

Cul2-TYPE UBIQUITIN LIGASE

$CRL2^{pVHL}$ COMPLEX

von Hippel–Lindau disease is a hereditary cancer syndrome caused by germline mutations in the *VHL* tumor suppressor gene (Latif

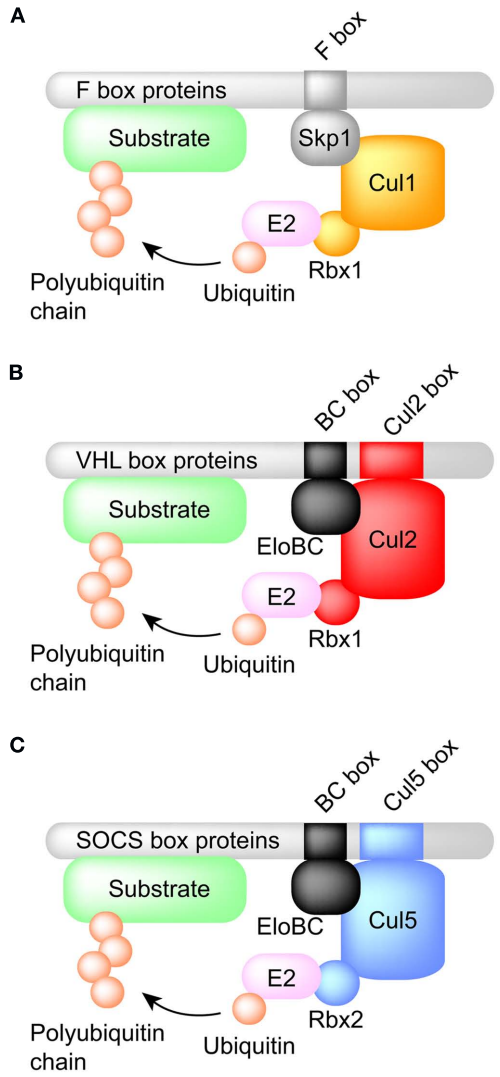


FIGURE 1 | Comparison of the structures of SCF- and ECS-type ubiquitin ligases. (A) SCF-type ubiquitin ligases. Cul1 is used as a scaffold protein and Skp1 bridges the gap between the F box protein and Cul1. **(B)** Cul2-type ubiquitin ligases. Cul2 is used as a scaffold protein and the Elongin BC complex connects the VHL box protein to Cul2. The Cul2 box determines the use of Cul2 as the scaffold protein. **(C)** Cul5-type ubiquitin ligases. Cul5 is used as a scaffold protein. Note that the Rbx2 is used for the recruitment of E2 enzyme.

et al., 1993). pVHL is the protein product of the *VHL* tumor suppressor gene and can bind to the Elongin BC complex. Elongin A and pVHL share a conserved Elongin C-binding sequence motif (S,T,P)LXXX(C,S,A)XXXΦ, which is referred to as the BC box (Conaway et al., 1998; Mahrour et al., 2008). More than 70% of VHL disease and sporadic clear cell renal carcinomas are caused by mutation or deletion of the BC box, which reduces binding affinity to the Elongin BC complex (Duan et al., 1995; Kishida et al., 1995). Approximately 57% of sporadic clear cell renal carcinomas contain inactivating mutations of VHL, of which 98% are caused by loss of heterozygosity (LOH) at the *VHL* locus (Gnarra et al., 1994). Epigenetic silencing of *VHL* by DNA methylation is also involved in the inactivation of VHL (Herman et al., 1996). Although pVHL inhibits the transcriptional activity of Elongin A by competing for binding sites on the Elongin BC complex (Duan et al., 1995), this review will focus on its ubiquitin ligase activity rather than its affect on Elongin-mediated transcription. In addition to the Elongin BC complex, the VHL complex also contains Cul2 and Rbx1 and is similar to SCF (Skp1–Cul1–F box protein) type ubiquitin ligases (**Figure 1**; Kibel et al., 1995; Pause et al., 1997; Kamura et al., 1999). In fact, the VHL complex has ubiquitin ligase activity and targets the hypoxia-inducible factor-α (HIF-α) family of transcription factors (HIF-1–3α) for proteasomal degradation (**Figure 2**; Maxwell et al., 1999). At normal oxygen levels, proline residues of the LXXLAP sequence motif within the oxygen-dependent degradation domain (ODDD) of HIF-α are hydroxylated and recognized by pVHL (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Hon et al., 2002). As a result, HIF-α is polyubiquitinated and degraded. Three HIF prolyl hydroxylases (PHD1–3) have been identified in mammals and shown to hydroxylate HIF-α subunits (Epstein et al., 2001). Since PHD2 is a critical enzyme for the hydroxylation of HIF-1α, PHD1, and 3 may hydroxylate other target substrates (Berra et al., 2003). In low oxygen conditions, PHDs are unable to hydroxylate the HIF-α subunits, which are therefore not recognized and targeted for degradation by pVHL. The unhydroxylated HIF-α dimerizes with constitutively expressed HIF-1β, also called aryl hydrocarbon receptor nuclear translocator (ARNT), and translocates to the nucleus, where it induces the transcription of downstream target genes, including vascular endothelial growth factor A (*VEGFA*), solute carrier family 2 member 1 (*SLC2A1*, which encodes GLUT1), and platelet-derived growth factor-β (*PDGFB*; Kourembanas et al., 1990; Wizigmann-Voos et al., 1995; Gnarra et al., 1996; Iliopoulos et al., 1996; Maxwell et al., 1999). Loss of functional pVHL protein prevents the O_2-dependent degradation of HIF-α, resulting in constitutive expression of HIF-dependent genes and consequently VHL disease.

The pVHL protein contains the recently defined "VHL box," which is composed of a BC box and a Cul2 box (**Figure 3**; Kamura et al., 2004). The Cul2 box specifically recognizes the endogenous Cul2/Rbx1 complex in a similar manner to SCF-type ubiquitin ligase recognition by F box and Skp1 (**Figure 1**; Kamura et al., 2004). The ring finger protein Rbx1 recruits ubiquitin-conjugating enzymes and is an essential molecule for the formation of SCF-type and Cul2-type ubiquitin ligases (Kamura et al., 1999, 2004; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999). Further study demonstrated that the Cul2 box is located 8–23 amino acids C-terminal to the BC box and has the consensus sequence ΦPXXΦXXXΦ, where the first position is most frequently a leucine (Mahrour et al., 2008). The Cul2 box is therefore partially similar to the Cul5 box.

pVHL also polyubiquitinates and induces the degradation of Sprouty2 (Spry2), which is implicated in the growth and progression of tumors (Anderson et al., 2011). Proline residues of Spry2 are hydroxylated by PHD at normoxia and are recognized by pVHL for polyubiquitination and degradation (Anderson et al., 2011). Epidermal growth factor receptor (EGFR) is also targeted by pVHL for polyubiquitination and degradation (Zhou and

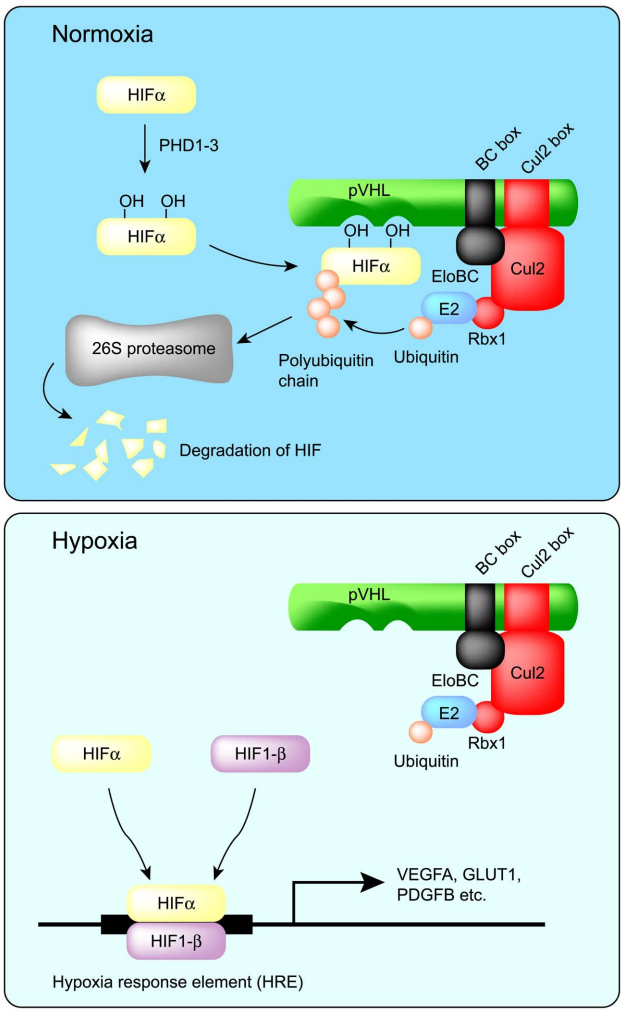


FIGURE 2 | Regulation of HIF-α protein by pVHL. In normoxia, two proline residues of HIF-α are hydroxylated by PHD1–3, and then HIF-α is recognized by pVHL for polyubiquitination and degradation by the proteasome. On the other hand, in hypoxia, HIF-α escapes prolyl hydroxylation and thereby escapes degradation. HIF-α then dimerizes with HIF-1β to form an active transcription complex. Prolonged activation of the HIFα/β transcription complex is a major contributor to VHL disease.

Yang, 2011). pVHL is proposed to down-regulate tumor growth caused by prolonged signaling of activated EGFR (Zhou and Yang, 2011). pVHL also mediates the polyubiquitination of the atypical PKCs, PKCλ, and PKCζII (Okuda et al., 2001; Iturrioz and Parker, 2007). PKCζII interacts with Par6, which plays a critical role in the development of tight junction structures and apicobasal polarization. It also inhibits tight junction formation and thereby plays a regulatory role in the development and transformation of cell polarity (Suzuki et al., 2001; Parkinson et al., 2004). PKCλ may have a similar function, which is also inhibited by pVHL through ubiquitin-dependent degradation. pVHL also polyubiquitinates the seventh subunit of human RNA polymerase II (hsRPB7) and suppresses hsRPB7-dependent VEGF promoter transactivation, VEGF mRNA expression, and VEGF protein secretion (Na et al., 2003). The large subunit of RNA polymerase II (Rpb1), which has sequence and structural similarity to the pVHL-binding domain of HIF-1α, is bound and polyubiquitinated by pVHL (**Figure 4**; Kuznetsova et al., 2003). The interaction between pVHL and Rpb1 is enhanced by hyperphosphorylation of Rpb1 by UV radiation, which indicates that Rpb1 ubiquitination may have a role in transcription-coupled DNA repair (**Figure 4**; Svejstrup, 2002; Kuznetsova et al., 2003). Further study showed that proline 1465 of Rpb1, which is located within the LXXLAP motif, is hydroxylated mainly by PHD1 during oxidative stress (Mikhaylova et al., 2008). pVHL is necessary for the oxidative stress-dependent hydroxylation of Pro1465, the phosphorylation of Ser5, and the polyubiquitination of Rpb1 and its recruitment to the DNA (Mikhaylova et al., 2008). Surprisingly, in renal clear cell carcinoma (RCC), pVHL increased the protein abundance and non-degradative ubiquitination of Rpb1 (Mikhaylova et al., 2008). Polyubiquitination of Rpb1 in RCC cells by pVHL contributes to tumor growth by modulating gene expression (Mikhaylova et al., 2008). This is different from previous results found in PC12 cells, in which pVHL polyubiquitinates Rpb1 for protein degradation (Kuznetsova et al., 2003). How pVHL differentially regulates Rpb1 in cells of different origins awaits further investigation.

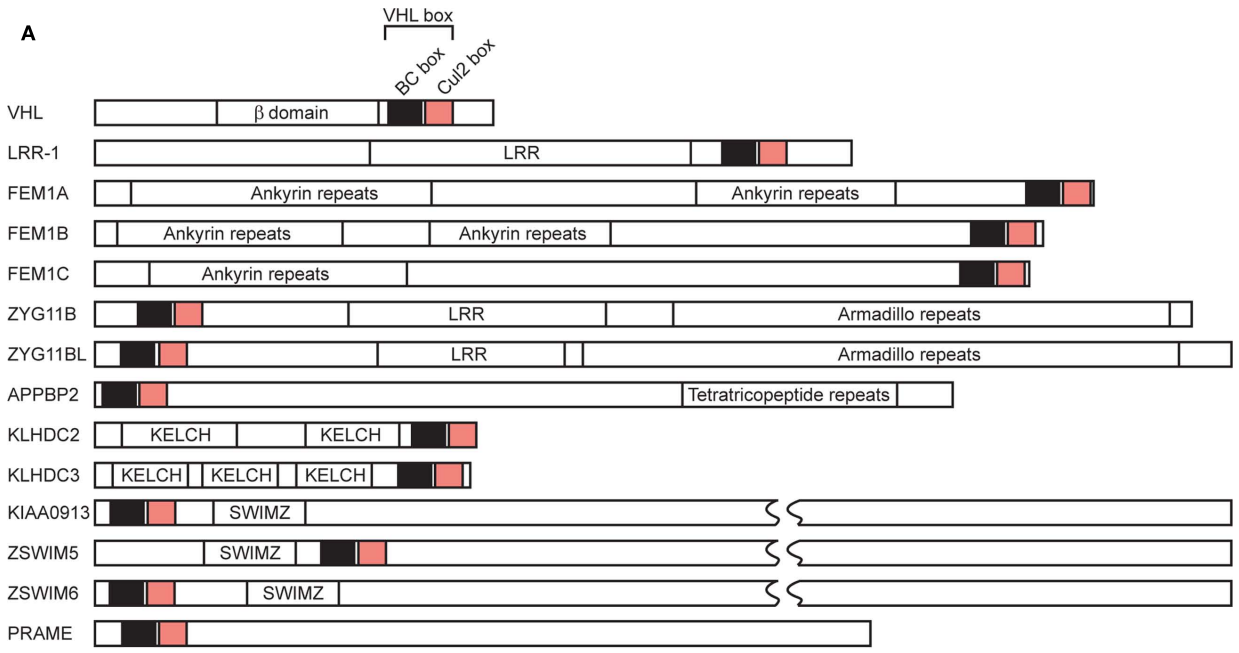
CRL2^{LRR-1} COMPLEX

Leucine-rich repeat protein (LRR)-1 contains a VHL box and physiologically interacts with the endogenous Cul2–Rbx1 complex (**Figure 3**; Kamura et al., 2004; Costessi et al., 2011). In fact, nematode LRR-1 degrades the Cip/Kip CDK-inhibitor CKI-1 in *C. elegans* to promote cell cycle progression in germ cells (Starostina et al., 2010). Human LRR-1 also polyubiquitinates the CDK-inhibitor p21^{Cip1}; however, it does not affect cell cycle progression (Starostina et al., 2010). Rather, human LRR-1 targets cytoplasmic p21 for degradation to prevent the inhibition of the Rho/ROCK/LIMK pathway (Starostina et al., 2010). These data indicate that human LRR-1 is a negative regulator of cofilin, an actin-depolymerizing protein that decreases cell motility (Starostina et al., 2010).

CRL2^{FEM1B} COMPLEX

Feminization-1 (FEM-1) also contains a VHL box and physiologically interacts with endogenous Cul2–Rbx1 complex (**Figure 3**). FEM-1 regulates apoptosis during the sex determination pathway of the nematode (Hodgkin et al., 1985). In *C. elegans*, FEM-1 interacts with CED-4, an Apaf-1 homolog, to promote apoptosis, suggesting an evolutionarily conserved role in apoptosis regulation (Chan et al., 2000). Nematode FEM-1 polyubiquitinates TRA-1, a Gli-family transcription factor and terminal effector of the sex determination pathway (Starostina et al., 2007). Mouse FEM-1 homolog B (FEM1B) interacts with and polyubiquitinates ankyrin repeat domain 37 (Ankrd37), which contains ankyrin repeats and a putative nuclear localization signal (NLS; Shi et al., 2011). Ankrd37 is highly enriched in mouse testis and is conserved from zebrafish to humans (Shi et al., 2011). These data indicate that the terminal step in sex determination is controlled by ubiquitin-mediated proteolysis.

Feminization-1 is polyubiquitinated by SEL-10, an F box and WD40 repeat protein, for proteasomal degradation (Jager



B

VHL	NP_000542	175	YRRLDIVRSLYEDLE 189
LRR-1	NP_689542	338	YGSHIIPFHLCQDLD 352
LRRC14	NP_055480	21	QALPLLPRELFPLLF 35
LRRC28	NP_653199	295	LSPISLPRSLLELLH 309
LRRC42	NP_443172	105	DSLIGFPEQIAEKLF 119
LRRC58	NP_001093148	277	YTPYDLPGNLLRYLG 291
FEM1A	NP_061178	653	PYKGFIPEDLEAFIE 667
FEM1B	NP_056137	611	NYQDQIPRTLEEFVG 625
FEM1C	NP_064562	600	YYKGHIPEKLETFVS 614
ZYG11B	NP_078922	45	QEPGVFPQEVADRLL 59
ZYG11BL	NP_006327	37	HPDIFLPSEICDRLV 51
APPBP2	NP_006371	30	RDIRSLPENIQFDVY 44
KLHDC2	NP_055130	381	NSWNCLPKHLLHSVN 395
KLHDC3	NP_476502	351	LDQSCLPHDIRWELN 365
KIAA0913	NP_055852	95	KVYPPVPEQLQLRIA 109
ZSWIM5	NP_065934	84	ERFERIPEPVQRRIV 98
ZSWIM6	NP_065979	96	ERFERIPEPVQRRIV 110
PRAME	NP_996839	43	AALELLPRELFPPLF 57
MED8	NP_963836	169	NKQTFNPTDTNALVA 183
HPV E7	NP_041326	64	TLRLCVQSTHVDIRT 78
Consensus			-----ΦP--Φ---Φ-

FIGURE 3 | Domain organization of VHL box proteins. (A) The VHL box consists of a BC box and a Cul2 box in the order indicated. LRR, leucine-rich repeats; SWIMZ, SWI2/SNF2 MuDR zinc fingers. **(B)** Alignment of amino acid sequences of selected Cul2 boxes. Identical amino acids are highlighted in yellow. GenBank™accession numbers of each protein are indicated. The consensus sequence is indicated below. Φ, hydrophobic residue.

et al., 2004). In mammalian cells, receptor for activated C kinase (RACK)1, also a WD40 repeat protein, associates with FEM1B and mediates the polyubiquitination and downregulation of FEM1B (Subauste et al., 2009). RACK1 also binds to the Elongin BC complex and promotes the ubiquitination of HIF-1α independently of the pVHL complex (Liu et al., 2007). Since the Elongin BC binding site in RACK1 is similar to that of pVHL, it has been suggested that RACK1 is a Cul2-type ubiquitin ligase (Liu et al., 2007).

CRL2^{PRAME} COMPLEX

Preferentially expressed antigen of melanoma (PRAME) contains a VHL box and physiologically interacts with endogenous Cul2–Rbx1 complex (Kamura et al., 2004; Costessi et al., 2011). Genome-wide chromatin immunoprecipitation experiments revealed that PRAME is specifically enriched at enhancers and at transcriptionally active promoters that are also bound by nuclear transcription factor Y (NFY), a transcription factor essential for early embryonic development (Bhattacharya et al., 2003; Costessi et al., 2011). However, the physiological substrates of PRAME have not yet been identified.

Cul5-TYPE UBIQUITIN LIGASE

CRL5$^{Cis/SOCS}$ COMPLEX

This family consists of suppressor of cytokine signaling (SOCS) proteins and cytokine-inducible Src homology 2 (SH2) domain-containing protein (CIS, also known as CISH), which

also interacts with the Elongin BC complex through its SOCS box (Piessevaux et al., 2008). To date, eight CIS/SOCS family proteins have been identified: CIS, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5, SOCS6, and SOCS7. All of them have a central SH2 domain as well as a C-terminally located SOCS box consisting of a 40-amino acid motif (**Figure 5**; Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Members of the CIS/SOCS family bind to janus kinases (JAKs), certain cytokine receptors, or signaling molecules, thereby suppressing downstream signaling events (Piessevaux et al., 2008). A small kinase inhibitory region (KIR) of SOCS1 and SOCS3 inhibits the JAKs by acting as a pseudo-substrate, resulting in the downregulation of further signal transduction (Piessevaux et al., 2008). The CIS/SOCS family can also down-regulate signaling by competing with downstream molecules for binding to the activated receptors (Ram and Waxman, 1999; Piessevaux et al., 2008) and can prevent signaling by polyubiquitination and degradation of target substrates. For example, SOCS1 polyubiquitinates JAK2, Vav, IRS1, and IRS2 (De Sepulveda et al., 2000; Kamizono et al., 2001; Rui et al., 2002). Recent studies also demonstrated that SOCS1 and SOCS3 are important regulators of adaptive immunity (Kile et al., 2002; Tamiya et al., 2011). Some SOCS box-containing proteins – for example, CIS, SOCS1–7, SPRY domain-containing SOCS box proteins (SSB1, SSB2, and SSB4, also known as SPSB1, 2, and 4, respectively), ras-related protein Rab-40C (also known as RAR3), WD40 repeat-containing SOCS box protein WSB1, leucine-rich repeat protein MUF1, and ankyrin repeat- and SOCS box-containing protein (ASB)11 – also contain a BC box and a Cul5 box inside the SOCS box (**Figure 5**; Hilton et al., 1998; Kamura et al., 2001, 2004; Babon et al., 2009; Sartori da Silva et al., 2010). The amino acid sequence LPΦP in the Cul5 box results in a specific interaction with Cul5, particularly when there is a proline in the fourth position of the motif (Kamura et al., 2004). Furthermore, endogenous Cul5 interacts with endogenous Rbx2, enabling SOCS box-containing proteins to form a protein complex with Cul5 and Rbx2 (**Figure 1C**; Kamura et al., 1999, 2004; Ohta et al., 1999). The selective interactions between Cul2 and Rbx1 or Cul5 and Rbx2 suggest that Rbx1 and Rbx2 are functionally distinct, at least in terms of their specific binding to Cullin family members. Although SOCS1 contains a Cul5 box, no interaction between SOCS1 and Cul5 has been detected, most likely because the Cul5 box is incompletely conserved (Kamura et al., 2004). Since SOCS1 polyubiquitinates JAK2, Vav, IRS1, and IRS2 (De Sepulveda et al., 2000; Kamizono et al., 2001; Rui et al., 2002), it is possible that the interaction of SOCS1 with these substrates recruits other ubiquitin ligase(s) that actually mediate their polyubiquitination and degradation, or that SOCS1 binds to the Cul5–Rbx2 module too weakly to have been previously detected (Kamura et al., 2004). Recently, it was reported that SOCS1 and SOCS3 bind more weakly to Cul5, with affinities of 100- and 10-fold lower, respectively, than to the rest of the family (Babon et al., 2009). In general, micromolar affinities are common in physiological interactions, and SOCS1 and SOCS3 have 1 and 0.1 μM affinities, respectively, for Cul5 (Babon et al., 2009). Therefore it is possible that all CIS/SOCS family members can act as ubiquitin ligases. This may explain why only SOCS1 and SOCS3 have been shown to suppress signaling using both SOCS box-dependent and -independent mechanisms (Babon et al., 2009).

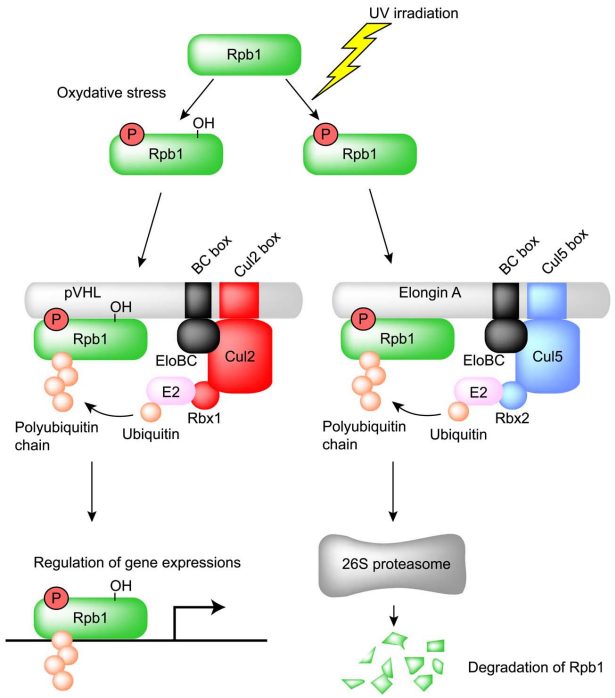


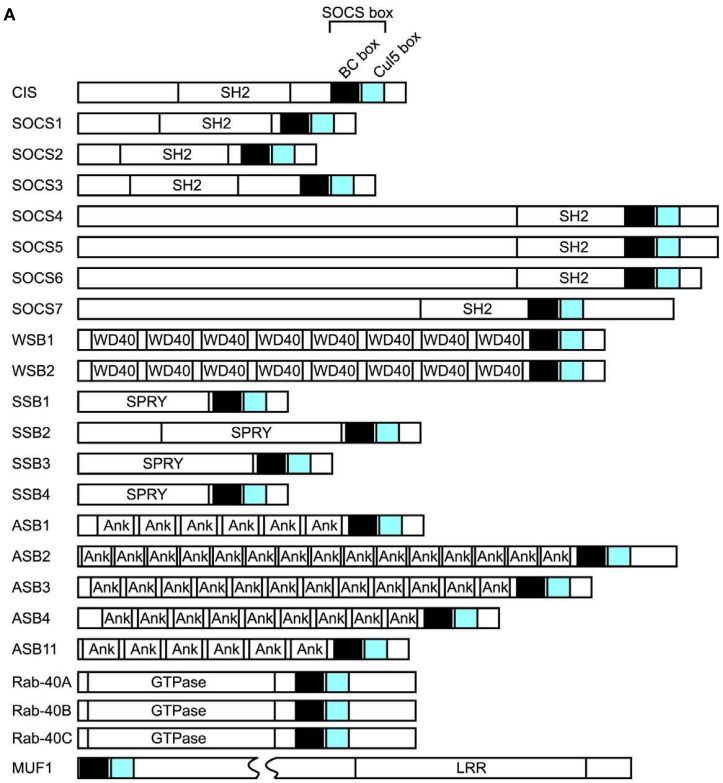
FIGURE 4 | Regulation of the large subunit of RNA polymerase II (Rpb1) by pVHL and Elongin A. Ser5 and Pro1465 of Rpb1 are phosphorylated and hydroxylated, respectively, by oxidative stress and then recognized by the Cul2-type ubiquitin ligase pVHL. The resulting polyubiquitinated Rpb1 is not degraded by proteasome and contributes to tumor growth by modulating gene expression in renal clear cell carcinoma. Ser5 of Rpb1 is also phosphorylated by UV irradiation and recognized by the Cul5-type ubiquitin ligase Elongin A for proteasomal degradation.

CRL5$^{Elongin\ A}$ COMPLEX

pVHL is a ubiquitin ligase for the large subunit of RNA polymerase II (Rpb1), as mentioned above. Interestingly, ubiquitination and proteasomal degradation of Rpb1 following UV irradiation are significantly suppressed in Elongin A-deficient cells, which suggests that Elongin A is also a ubiquitin ligase for Rpb1 (Yasukawa et al., 2008). In fact, polyubiquitination and degradation are rescued by the transfection of wild-type Elongin A (Yasukawa et al., 2008). Furthermore, Elongin A and the Elongin BC complex can associate with Cul5 and Rbx2, and this complex efficiently polyubiquitinates Rpb1 *in vitro* (Yasukawa et al., 2008). Phosphorylation of Rpb1 at Ser5 after UV irradiation significantly enhanced the interaction between Elongin A and Rpb1 (Yasukawa et al., 2008). These data indicate that Elongin A, like pVHL, is involved in the ubiquitination and degradation of Rpb1 following DNA damage (**Figure 4**).

CRL5^{SSB} COMPLEX

Inducible nitric oxide (NO) synthase (iNOS, NOS2) is a high-output NOS compared with NOS1 and NOS3. The activity of iNOS is approximately 10-fold greater than that of NOS1 and NOS3 (Lowenstein and Padalko, 2004). iNOS is not expressed under normal conditions but is induced in response to cytokines, microbes, or microbial products, resulting in the sustained



B

CIS	NP_037456	255	VDCLPLPRRMADYLR 269
SOCS1	NP_003736	191	LARIPLNPVLRDYLS 205
SOCS2	NP_003868	178	IWGLPLPTRLKDYLE 192
SOCS3	NP_003946	207	VTQLPGPIREFLDQY 221
SOCS4	NP_955453	406	IDALPIPSSMKLYLK 420
SOCS5	NP_659198	501	IDGLPLPSMLQDFLK 505
SOCS6	NP_004223	517	IQKLPLPNKMKDYLQ 531
SOCS7	NP_055413	535	IPDLPLPKPLISYIR 549
WSB1	NP_056441	404	VQELPIPSKLLEFLS 418
WSB2	NP_061109	386	VLALPIPKKMKEFLT 400
SSB1	NP_079382	257	IHTLPLPASLKAYLL 271
SSB2	NP_001139788	247	VSALPLPPAMKRYLL 261
SSB3	NP_543137	296	LEGLPLPPGLKQVLH 310
SSB4	NP_543138	257	ISSLPLPQSLKNYLQ 271
ASB1	NP_057198	319	IPSLPLPDPIKKFLL 333
ASB2	NP_001189358	616	IDTLPLPGRLIRYLK 630
ASB3	NP_057199	485	ISQLPLPRSLHNYLL 499
ASB4	NP_057200	406	LLSLPLSLKKYLLLE 420
ASB5	NP_543150	313	IPQLQLPTLLKNFLQ 327
ASB6	NP_060343	396	VKALPLPDRLKWYLL 410
ASB7	NP_937886	298	LDELPIAKVMKDYLK 312
ASB8	NP_077000	271	VKGLPLPASLKEYLL 285
ASB9	NP_001026909	278	ITKLVLPEDLKQFLL 292
ASB10	NP_001135931	445	LPRLPLPPRLLRYLQ 459
ASB11	NP_543149	307	IHKLHLPEPLERFLL 321
ASB12	NP_569059	301	INQLDIPPMLISYLK 315
ASB13	NP_078977	262	IAKLNIPPRLIDYLS 276
ASB14	NP_001136205	557	MSFLPLPNRLKAYVL 571
ASB15	NP_563616	560	VEKLPLPPAIQRYIL 574
ASB16	NP_543139	431	ATRLPLPPLLRDYLL 445
ASB17	NP_543144	278	IFSLLIPARLQNYLN 292
ASB18	NP_997721	444	IPLLPLPKPLQNYLL 458
Rab-40A	NP_543155	209	VDKLPLPSTLRSHLK 223
Rab-40B	NP_006813	209	VDKLPLPIALRSHLK 223
Rab-40C	NP_066991	209	IDKLPLPVTIKSHLK 223
MUF1	NP_006360	65	VWALPGPILQSILPL 79
EloA	NP_003189	593	FEVGGVPYSVLEPVL 607
Consensus			Φ--LPΦP--Φ--YL- F

FIGURE 5 | Domain organization of SOCS box proteins. (A) The SOCS box consists of a BC box and a Cul5 box in the order indicated. SH2, Src homology 2 phosphotyrosine binding domain; WD40, WD40 repeats; SPRY, sp1A/ryanodine receptor domain; Ank, ankyrin repeats; LRR, leucine-rich repeats; GTPase, GTPase domain. **(B)** Alignment of amino acid sequences of selected Cul5 boxes. Identical amino acids are highlighted in yellow. GenBank™accession numbers of each protein are indicated. The consensus sequence is indicated below. Φ, hydrophobic residue.

production of NO (Lowenstein and Padalko, 2004). As a result, reactive nitrogen intermediates (such as NO, nitrite, and nitrate) and the products of the interaction of NO with reactive oxygen species (such as peroxynitrite and peroxynitrous acid) are accumulated and used to inhibit viruses or bacteria (Fang, 1997; Nathan and Shiloh, 2000; Lowenstein and Padalko, 2004). SSB1, 2 and 4 polyubiquitinate iNOS for proteasomal degradation (Kuang et al., 2010; Nishiya et al., 2011). SSB2-deficient macrophages showed prolonged iNOS and NO production, resulting in the enhanced killing of *L. major* parasites (Kuang et al., 2010). Further study showed that SSB1 and SSB4 are major ubiquitin ligases for iNOS and prevent the overproduction of NO, which could cause cytotoxicity (Nishiya et al., 2011).

CRL5^{WSB1} COMPLEX

WSB1 polyubiquitinates homeodomain-interacting protein kinase 2 (HIPK2), which is a nuclear protein kinase and is well-conserved from *Drosophila* to humans (Choi et al., 2005, 2008). HIPK2 interacts with a variety of transcription factors (D'Orazi et al., 2002; Hofmann et al., 2002; Zhang et al., 2005; Kim et al., 2006), the p300/CBP co-activator (Kim et al., 2002; Aikawa et al., 2006), and the Groucho/TLE co-repressor (Choi et al., 2005). The loss of HIPK2 reduces apoptosis and increases the numbers of trigeminal ganglia, while the overexpression of HIPK2 in the developing sensory and sympathetic neurons promotes apoptosis in a caspase-dependent manner (Doxakis et al., 2004; Wiggins et al., 2004). HIPK2 plays an important role in apoptosis mediated by p53, CtBP, Axin, Brn3, Sp100, TP53INP1, and PML (Moller et al., 2003a,b; Tomasini et al., 2003; Doxakis et al., 2004; Kanei-Ishii et al., 2004). UV irradiation activates and stabilizes HIPK2, most likely by WSB1-independent auto-phosphorylation, which results in the phosphorylation of p53 at Ser46. Expression of p53 target genes then promotes apoptosis (D'Orazi et al., 2002; Hofmann et al., 2002). Genotoxic stresses, such as adriamycin and cisplatin, also inhibit polyubiquitination of HIPK2 by WSB1 (Choi et al., 2008). HIPK2 also phosphorylates CtBP at Ser422 and phosphorylated CtBP is degraded via the 26S proteasome, resulting in apoptosis in p53-deficient cells (Zhang et al., 2003). WSB1 expression is induced by Sonic hedgehog (Shh) in developing limb buds and other embryonic structures (Vasiliauskas et al., 1999). WSB1 also ubiquitinates the thyroid hormone-activating enzyme type 2 iodothyronine deiodinase (D2; Dentice et al., 2005). Ubiquitination of Shh-induced D2 by WSB1 induces parathyroid hormone-related peptide (PTHrP), thereby regulating chondrocyte differentiation (Dentice et al., 2005). In addition to HIPK2 and D2, WSB1 also binds to the interleukin-21 receptor (IL-21R; Nara et al., 2011). However, instead of promoting its degradation, WSB1 inhibits the degradation of the mature form of IL-21R (Nara et al., 2011). WSB1 associates with the intracytoplasmic region of IL-21R and enhances the maturation of IL-21R from an N-linked glycosylated form to a fully glycosylated mature form (Nara et al., 2011). These data indicate that WSB1 has important roles in both the maturation and the degradation of IL-21R.

CRL5^{ASB} COMPLEX

ASB2, 3, 4, 6, 9, and 11 can all bind to Cul5–Rbx2 and form ubiquitin ligase complexes. Retinoic acid induces ASB2 in acute promyelocytic leukemia cells (Guibal et al., 2002). ASB2 targets the actin-binding proteins filamin A and B for proteasomal degradation (Heuze et al., 2008). Since knockdown of endogenous ASB2 in leukemia cells delays retinoic acid-induced differentiation and filamin degradation, ASB2 may regulate hematopoietic cell differentiation by targeting filamins for degradation and thereby modulating actin remodeling (Heuze et al., 2008). ASB2 and Skp2 associate with each other to bridge the formation of a non-canonical cullin1- and cullin5-containing dimeric ubiquitin ligase complex and promote the polyubiquitination and degradation of Jak3 (Nie et al., 2011; Wu and Sun, 2011).

Tumor necrosis factor receptor type 2 (TNF-R2) is polyubiquitinated by ASB3 for proteasomal degradation (Chung et al., 2005). ASB3 can affect T cell signaling by degrading TNF-R2, resulting in the inhibition of downstream signaling events in response to TNF-α (Chung et al., 2005).

Insulin receptor substrate 4 (IRS4) is an adaptor molecule involved in signal transduction by both insulin and leptin, and is widely expressed throughout the hypothalamus, with the greatest expression observed in the medial preoptic nucleus, ventromedial hypothalamus, and arcuate nucleus (Numan and Russell, 1999). ASB4 co-localizes and interacts with IRS4 in hypothalamic neurons (Li et al., 2011). ASB4 polyubiquitinates IRS4 for degradation and decreases insulin signaling (Li et al., 2011).

ASB6 is expressed in 3T3-L1 adipocytes but not in fibroblasts (Wilcox et al., 2004). ASB6 may regulate components of the insulin signaling pathway in adipocytes by promoting the degradation of adapter protein with a pleckstrin homology and SH2 domain (APS; Wilcox et al., 2004).

ASB9 polyubiquitinates creatine kinase B (CKB) and decreases total CKB levels (Debrincat et al., 2007).

The notch signaling pathway is essential for the spatio-temporal regulation of cell fate (Mumm and Kopan, 2000; Lai, 2004; Louvi and Artavanis-Tsakonas, 2006). The single-pass transmembrane protein delta acts as a ligand for the notch receptor. *Danio rerio* Asb11 (d-Asb11) regulates compartment size in the endodermal and neuronal lineages via the ubiquitination and degradation of deltaA, leading to the activation of the canonical notch pathway (Diks et al., 2006, 2008). This recognition is specific to deltaA because d-Asb11 does not degrade deltaD (Diks et al., 2008). In zebrafish embryos, knockdown of d-Asb11 repressed specific delta–notch elements and their transcriptional targets, whereas these were induced when d-Asb11 was misexpressed (Diks et al., 2008). These data indicate that d-Asb11 regulates delta–notch signaling for the fine-tuning of lateral inhibition gradients between deltaA and notch (Diks et al., 2008).

CRL5$^{RAB-40C}$ COMPLEX AND CRL^{MUF1} COMPLEX

The substrates of Rab-40C and MUF1 have not yet been identified. However, Rab-40C localizes in the perinuclear recycling compartment, suggesting its physiological role in receptor endocytosis (Rodriguez-Gabin et al., 2004). Given that the mRNA and protein level of Rab-40C increases as oligodendrocytes differentiate, it may be important in myelin formation (Rodriguez-Gabin et al., 2004).

VIRAL ECS-TYPE UBIQUITIN LIGASE

CRL2^{HPV16E7} COMPLEX

Human papillomavirus (HPV) type 16 cause premalignant squamous intraepithelial neoplasia (Munger et al., 2004). Integration of viral DNA into the host genome leads to persistent and dysregulated expression of HPV E6 and E7 oncoproteins, which is necessary for the induction and maintenance of the oncogenic transformation (Munger et al., 2004). HPV E7 contains incomplete Cul2 box and can bind to endogenous Cul2 (Huh et al., 2007). HPV E7 polyubiquitinates retinoblastoma tumor suppressor (pRB) and induces proteasomal degradation (Boyer et al., 1996; Berezutskaya et al., 1997; Jones and Munger, 1997; Huh et al., 2007).

CRL5^{Vif} COMPLEX

The viral infectivity factor (Vif) protein of human immunodeficiency virus-1 (HIV-1) is also a Cul5-type ubiquitin ligase (Yu et al., 2003; Bergeron et al., 2010). Importantly, it has been suggested that the zinc-binding motif of Vif is important for its interaction with Cul5 (Yu et al., 2004; Mehle et al., 2006; Xiao et al., 2006). Vif polyubiquitinates and degrades the cellular intrinsic restriction factors APOBEC3F and APOBEC3G (Yu et al., 2003; Mehle et al., 2004; Liu et al., 2005). Both APOBEC3F and G have cytidine deaminase activity and, when packaged into HIV-1 virions, cause uracil (U) to be substituted for cytosine (C) in newly synthesized minus-strand viral DNA (Sheehy et al., 2002; Harris et al., 2003; Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003). The C-to-U mutation introduced into minus-strand viral DNA results in a guanine (G)-to-adenine (A) mutation in plus-strand viral DNA because U is read as T by DNA polymerases (Lecossier et al., 2003). These mutations cause amino acid substitutions, which affect the enzymatic activity of HIV-1 (Harris et al., 2003). Another possibility is that deoxyuridine in minus-strand viral DNA is targeted for excision by uracil-DNA glycosylase (Harris et al., 2003). These abasic sites are recognized and cleaved by endonucleases, inhibiting HIV-1 replication (Harris et al., 2003). Since the CRL5^{Vif} complex targets APOBEC3F and APOBEC3G for proteasomal degradation, it is a potential target for the development of antiviral agents aimed at preventing the interaction between Vif and Cul5.

CRL5^{E4orf6} COMPLEX

The human adenovirus type 5 (Ad5) early region 4 34-kDa product from open reading frame 6 (E4orf6) contains three BC boxes (Blanchette et al., 2004; Cheng et al., 2007, 2011). Although Ad5 E4orf6 forms complex containing Cul5, Elongin BC complex, and Rbx1, Cul5 box is not present in the Ad5 E4orf6 (Harada et al., 2002; Blanchette et al., 2004; Cheng et al., 2011). Adenoviral protein E1B55K associates with the E4orf6 protein and recognizes substrate to be degraded by ubiquitin–proteasome pathway (Blanchette et al., 2004; Cheng et al., 2007; Luo et al., 2007). This complex is essential for efficient viral replication and some substrates have been identified, including p53 (Moore et al., 1996; Querido et al., 1997; Steegenga et al., 1998; Cathomen and Weitzman, 2000; Nevels et al., 2000; Shen et al., 2001), meiotic recombination 11 (Mre11; Stracker et al., 2002; Blanchette et al., 2004), DNA ligase IV (Baker et al., 2007), integrin α3 (Dallaire et al., 2009), and adeno-associated virus type 5 (AAV5) Rep52 and capsid proteins (Nayak et al., 2008). The Mre11 complex consists of Mre11, RAD50, and Nijmegen breakage syndrome 1 (NBS1, also known as nibrin) is a sensor of DNA double-strand breaks (DSBs) and induces p53-dependent apoptosis (Stracker and Petrini, 2011). DNA ligase IV plays a pivotal role in repairing DSBs and the mutation of this gene results in ligase IV (LIG4) syndrome characterized by pronounced radiosensitivity, genome instability, malignancy, immunodeficiency, and bone marrow abnormalities (Chistiakov et al., 2009). Heterodimer of integrin α and β subunits functions as transmembrane receptor that links external ligands to intracellular signaling pathways. Integrin α3β1 heterodimer in which the α3 subunit is coupled to the β1 subunit binds a variety of extracellular matrix substrates, including fibronectin, collagen, vitronectin, and laminins (DiPersio et al., 1995). E4orf6/E1B55K ligase complex is

Table 1 | Cul2-type ubiquitin ligases and corresponding substrates.

Ubiquitin ligase	Substrates	References
pVHL	HIFα	Ivan et al. (2001); Jaakkola et al. (2001); Masson et al. (2001); Hon et al. (2002)
	Spry2	Anderson et al. (2011)
	EGFR	Zhou and Yang (2011)
	Atypical PKC (PKCλ and ζII)	Okuda et al. (2001); Iturrioz and Parker (2007)
	RPB7	Na et al. (2003)
	Rpb1	Kuznetsova et al. (2003); Mikhaylova et al. (2008)
LRR-1	CKI-1 (in *C. elegans*)	Starostina et al. (2010)
	p21^{Cip}	Starostina et al. (2010)
FEM1B	TRA-1	Starostina et al. (2007)
	Ankrd37	Shi et al. (2011)

Table 2 | Cul5-type ubiquitin ligases and corresponding substrates.

Cul5-type ubiquitin ligases	Substrates	References
SOCS1	JAK2	Kamizono et al. (2001)
	Vav	De Sepulveda et al. (2000)
	IRS1 and IRS2	Rui et al. (2002)
ElonginA	Rpb1	Yasukawa et al. (2008)
SSB1, 2, and 4	iNOS	Kuang et al. (2010); Nishiya et al. (2011)
WSB1	HIPK2	Choi et al. (2005, 2008)
	D2	Dentice et al. (2005)
ASB2	Filamin A and B	Heuze et al. (2008)
	Jak3	Nie et al. (2011); Wu and Sun (2011)
ASB3	TNF-R2	Chung et al. (2005)
ASB4	IRS4	Li et al. (2011)
ASB6	APS	Wilcox et al. (2004)
ASB9	CKB	Debrincat et al. (2007)
ASB11	DeltaA (in *Danio rerio*)	Diks et al. (2006, 2008)

Table 3 | Viral ECS-type ubiquitin ligases and corresponding substrates.

Viral ECS-type ubiquitin ligases	Substrates	References
HPV16E7 (Cul2-type)	pRB	Boyer et al. (1996); Berezutskaya et al. (1997); Jones and Munger (1997); Huh et al. (2007)
Vif (Cul5-type)	APOBEC3F and APOBEC3G	Yu et al. (2003); Mehle et al. (2004); Liu et al. (2005)
E4orf6 of Ad5 (Cul5-type)	p53	Moore et al. (1996); Querido et al. (1997); Steegenga et al. (1998); Cathomen and Weitzman (2000); Nevels et al. (2000); Shen et al. (2001)
	Mre11	Stracker et al. (2002); Blanchette et al. (2004)
	DNA ligase IV	Baker et al. (2007)
	Integrin α3	Dallaire et al. (2009)
	AAV5 Rep52 and capsid proteins	Nayak et al. (2008)
E4orf6 of Ad16 (Cul2 and Cul5-type)	DNA ligase IV	Cheng et al. (2011)
BZLF1 (Cul2 and Cul5-type)	p53	Sato et al. (2009a,b)

involved in cell detachment from the extracellular matrix, which may contribute to virus spread (Dallaire et al., 2009). Although Cul5 is present in the E4orf6 complex of the human Ad5, Cul2 is primarily present in the E4orf6 complex of Ad12 and Ad40 (Cheng et al., 2011). Interestingly, E4orf6 complex of Ad16 binds Cul2 as well as Cul5 and is not able to degrade p53 and integrin α3 (Cheng et al., 2011). It remains unclear how E4orf6 complexes of each serotypes distinguish Cul2 from Cul5.

CRL5^{BZLF1} COMPLEX

Epstein–Barr virus (EBV), a human γ-herpesvirus, is associated with several B cell and epithelial cell malignancies and there are two different infection states, latent, and lytic (Tsurumi, 2001). BZLF1 (known as Zta, EB1, or ZEBRA), is a transcriptional transactivator that induces EBV early gene expression to activate an EBV lytic cycle cascade (Chevallier-Greco et al., 1986; Countryman et al., 1987; Hammerschmidt and Sugden, 1988; Sinclair et al., 1991). BZLF1 can bind to Cul2 and Cul5 because of presence of both Cul2 and Cul5 boxes (Sato et al., 2009a). BZLF1 polyubiquitinates and induces degradation of p53 (Sato et al., 2009a,b). The degradation of p53 prevents apoptosis and is required for the efficient viral propagation in the lytic replication.

CONCLUSION

The "classical" SOCS box proteins can be divided into two distinct families. Cul2 and Cul5 within the VHL box and SOCS box, respectively, determine the association with Rbx1 or Rbx2. Given that Rbx1 and Rbx2 specifically interact with Cul2 and Cul5, respectively, the functions of Rbx1 and Rbx2 are different from each other, at least in higher eukaryotes. Cul2- and Cul5-type ubiquitin ligases are structurally similar because they have the Elongin BC complex adaptor protein and Cullin scaffold protein in common. As with other ubiquitin ligases, these two have various substrates and physiological functions (**Tables 1**, **2**, and **3**) and may have arisen independently during evolution.

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The roles of VHL-dependent ubiquitination in signaling and cancer

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The function of tumor suppressor VHL is compromised in the vast majority of clear cell renal cell carcinoma, and its mutations or loss of expression was causal for this disease. pVHL was found to be a substrate recognition subunit of an E3 ubiquitin ligase, and most of the tumor-derived mutations disrupt this function. pVHL was found to bind to the alpha subunits of hypoxia-inducible factor (HIF) and promote their ubiquitination and proteasomal degradation. Proline hydroxylation on key sites of HIFα provides the binding signal for pVHL E3 ligase complex. Beside HIFα, several other VHL targets have been identified, including activated epidermal growth factor receptor (EGFR), RNA polymerase II subunits RPB1 and hsRPB7, atypical protein kinase C (PKC), Sprouty2, β-adrenergic receptor II, and Myb-binding protein p160. HIFα is the most well studied substrate and has been proven to be critical for pVHL's tumor suppressor function, but the activated EGFR and PKC and other pVHL substrates might also be important for tumor growth and drug response. Their regulations by pVHL and their relevance to signaling and cancer are discussed.

Keywords: ubiquitin, VHL, HIF, ccRCC, EGFR, proline hydroxylation

von HIPPEL–LINDAU (*VHL*), THE REGULATION OF THE ALPHA SUBUNITS OF HYPOXIA-INDUCIBLE FACTOR, AND OXYGEN SENSING

Loss of function of the tumor suppressor gene VHL is causal in the pathogenesis of clear cell renal cell carcinoma (ccRCC). The vast majority (70–80%) of sporadic RCCs are pathologically characterized as ccRCC. Among them, approximately 70% harbor biallelic inactivation of *VHL* through mutation, deletion, or hypermethylation of promoter (Kaelin, 2002; Linehan and Zbar, 2004). Inherited germline mutations in *VHL* predispose these patients to bilateral kidney cancer earlier than the sporadic kidney cancer patients, since the loss of the remaining wild type allele occurs more readily than the loss of two alleles. The protein product of the *VHL* tumor suppressor gene, pVHL, is found to be the substrate recognition unit of an E3 ubiquitin ligase complex that contains Cullin 2 (Cul2), Elongin B and C, and Rbx1 (Kamura et al., 1999a,b). Interestingly, tumor-derived point mutations were found to cluster around substrate recognizing (β domain) or the Elongin C-binding (α domain) sites (Stebbins et al., 1999), stressing the importance of ubiquitin ligase activity to pVHL's tumor suppressor function. This complex targets the α subunits of the heterodimeric transcription factor hypoxia-inducible factor (HIF) for ubiquitination and proteasome-mediated degradation (Ohh et al., 2000). In addition to being a part of an E3 ubiquitin ligase complex, pVHL also regulates other HIF-independent biological processes such as inhibition of NF-κB activity (Yang et al., 2007), maintenance of chromosome stability (Thoma et al., 2009), and promoting cilia production (Schraml et al., 2009), which will not be reviewed in this article.

The best-characterized substrates for pVHL-containing ubiquitin ligase are the alpha subunits of the HIF transcription factor. HIF contains two subunits: the oxygen-sensitive alpha subunits (HIF1α, HIF2α, and HIF3α, for the simplicity they will be collectively called HIFα) and the constitutively expressed HIF1β subunit [also called the aryl hydrocarbon nuclear translocator (ARNT); Semenza, 2007]. pVHL recognizes the HIFα only after they are hydroxylated on either of two critical prolyl residues by members of the EglN family (also called PHDs or HPHs; Epstein et al., 2001; Ivan et al., 2001, 2002; Jaakkola et al., 2001). These enzymes require molecular oxygen, Fe(II) and 2-oxoglutarate for activity. Under normal oxygen tension (normoxia), the critical proline residues on HIFα subunits are hydoxylated (P402 and 564 on HIF1α), recognized by pVHL, poly-ubiquitinated, and destroyed by the proteasome. When the oxygen is deprived (hypoxia) by physiological or pathological conditions, the HIFα subunits will be produced but cannot be prolyl hydroxylated. They escape the recognition by pVHL, accumulate, and hetero-dimerize with HIF1β. The heterodimer enters nucleus, recruit transcriptional coactivator complexes (Arany et al., 1996; Ema et al., 1999), and regulate the expression of (inducing or suppressing) hundreds of target genes by binding to the hypoxia-response element (HRE; Semenza, 2003; **Figure 1**). Activation of HIF leads to physiological adaptations to the deprivation of oxygen: a metabolic shift to anaerobic glycolysis, increased secretion of pro-angiogenesis factors that leads to growth of blood vessels and increased blood supply, remodeling of the extracellular matrix, and resistance to apoptosis and increased mobility. In *VHL*-defective ccRCC tumors, enhanced angiogenesis and constitutive activation of the HIF pathway are

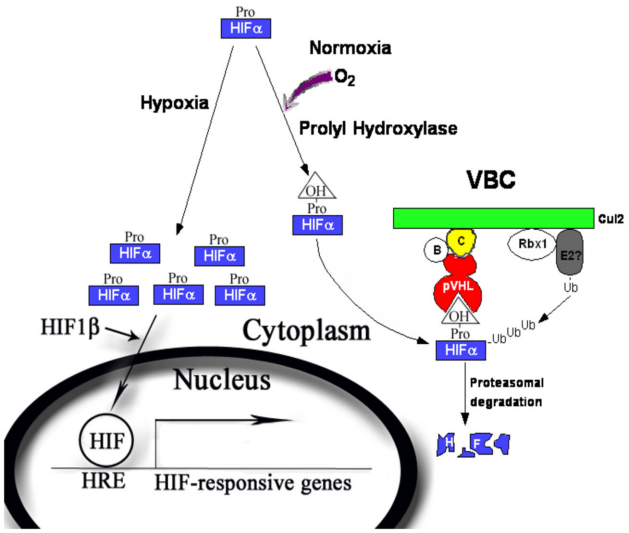

FIGURE 1 | The regulation of the HIFα by pVHL-containing E3 ubiquitin ligase complex. During normoxia, HIFα is produced and prolyl hydroxylated by PHD1–3. The hydroxyproline provides a binding signal for pVHL, which leads to efficient ubiquitylation and proteasomal degradation of HIFα protein. During hypoxia, HIFα is not prolyl hydroxylated and escapes pVHL recognition. HIFα accumulates and forms complex with HIF1β, goes into nucleus and turns on a transcriptional program to cope with the short-term and long-term effects of oxygen deprivation.

prominent features even when the oxygen supply is not limited. In the xenograft models of ccRCC, constitutive HIF activation was both sufficient (Kondo et al., 2002) and necessary for tumor growth (Kondo et al., 2003; Zimmer et al., 2004). In the clinic trials, drugs that block the activities of the receptors for vascular endothelial growth factor (VEGF), a critical HIF target gene, produced clear and positive, albeit often transient, clinical outcomes in kidney cancer patients (Rini, 2005).

Interestingly, although HIF2α is a potent oncogene, the activations of HIF targets are not necessarily all tumor-promoting events. HIF-dependent activation of REDD1 suppressed mTORC1 (Kucejova et al., 2011), and HIF-dependent activation of JARID1C decreased the overall level of the trimethylated histone H3 lysine 4 (H3K4Me3; Niu et al., 2012). Both were tumor-suppressive events, and kidney tumors found clever ways to inactivate them (Dalgliesh et al., 2010; Kucejova et al., 2011). Further careful analysis of how HIF targets contribute to kidney tumor growth and maintenance might yield new ways to treat kidney cancer.

ACTIVATED EPIDERMAL GROWTH FACTOR RECEPTOR

It is known that HIF can enhance epidermal growth factor receptor (EGFR) activity to promote tumor growth (de Paulsen et al., 2001; Franovic et al., 2007). In *VHL*-defective ccRCC cells, the expression of transforming growth factor-α (TGF-α), an agonist to EGFR, is induced by HIF2α. This stimulates cell proliferation through an autocrine loop (de Paulsen et al., 2001). At the same time, constitutively active HIF2α also increases the translational efficiency of EGFR mRNA (Franovic et al., 2007). Increased EGFR expression and elevated TGF-α work together to promote autonomous growth (cellular growth in the absence of stimulating growth factors), which is a hallmark of cancer. Stable suppression of EGFR by shRNAs prevents serum-free growth of *VHL*-defective ccRCC cells *in vitro*, and retards the tumor growth of these cells for extended periods *in vivo* without affecting HIF2α functions (Smith et al., 2005; Lee et al., 2008). This suggests that EGFR is critical for the tumor growth of *VHL*-defective ccRCC cells and could be a good therapeutic target in kidney cancer.

Epidermal growth factor receptor is implicated in many human cancers, as activating mutations of EGFR have been identified in human glioblastoma, non-small cell lung carcinomas (NSCLC), and colon cancer. Upon ligand binding, EGFR and its family members homo- or hetero-dimerize, *trans*-phosphorylate the c-terminal tyrosine residues. These phosphorylated residues recruit signaling molecules, which activate downstream effectors and elicit biological responses (Yarden and Sliwkowski, 2001). Ras/Raf/MEK/ERK and PI3K/PDK1/Akt1 are two major downstream pathways of activated EGFR. Since they promote both cellular proliferation and resistance to apoptosis (Jorissen et al., 2003), failure to turn off the activated EGFR can drive tumorigenesis.

Endocytosis and lysosome-mediated degradation is reported to be the major mechanism to down-regulate the activated EGFR. By binding to EGFR either directly through phosphorylated Y1045 (Levkowitz et al., 1999) or through its association with another EGFR-interacting protein Grb2 (Waterman et al., 2002), the ubiquitin ligase c-Cbl promotes its ubiquitination (Levkowitz et al., 1998). c-Cbl promotes mono-ubiquitylation on multiple lysine residues of EGFR, which is sufficient for EGFR endocytosis and degradation (Haglund et al., 2003a,b; Mosesson et al., 2003), However, mass-spectrometric and western blot analyses have suggested that a fraction of activated EGFR is poly-ubiquitinated (Huang et al., 2006; Umebayashi et al., 2008). Thus it is possible that other E3 ubiquitin ligases add poly-ubiquitin to the activated EGFR to promote its turnover.

Recently it was reported that pVHL was essential for the clearance of activated EGFR (Wang et al., 2009) and the proposed mechanism was that constitutively active HIF suppressed the lysosomal-mediated degradation of the activated EGFR. Specifically, Wang et al. suggested that HIF reduced the expression of Rabaptin-5. As Rabaptin-5 was critical for Rab5-mediated endosome fusion, reduced expression of Rabaptin-5 led to delayed EGFR sorting to the late endosome and lysosome, and this led to longer half-lives of the activated EGFR. This explanation predicted that delayed turnover of activated EGFR in *VHL*-defective ccRCC cells was due to high levels of HIF α subunits. However, Zhou and Yang (2011) found that the endogenous HIF was not the only or major cause of delayed EGFR turnover in *VHL*-defective ccRCC cells. Furthermore, they found that pVHL-mediated down-regulation of the activated EGFR was mostly mediated by proteasome instead of lysosome. In addition, loss of both c-Cbl and VHL caused the activated EGFR to become completely stable during the experiment, suggesting that these ubiquitin ligases collaborated to down-regulate activated EGFR. Finally it was reported that pVHL promoted the poly-ubiquitination of the activated EGFR, and this persisted in the absence of c-Cbl. Thus in ccRCC cells, pVHL promotes the poly-ubiquitination of the activated EGFR

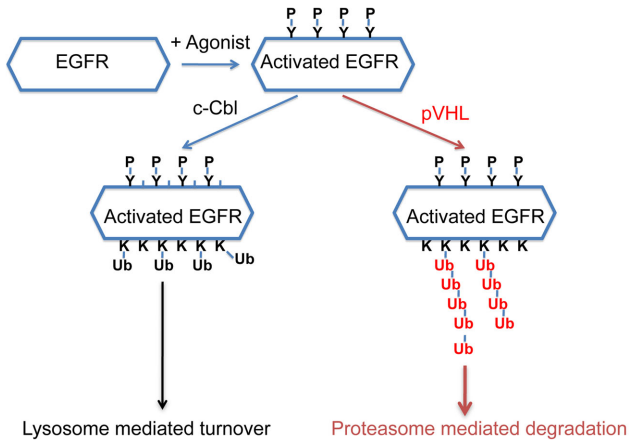

FIGURE 2 | A model of how pVHL and c-Cbl promote the turnover of the activated EGFR. After EGFR is activated by its agonist, it becomes phosphorylated at multiple sites. c-Cbl promotes multi-mono-ubiquitylation on the activated EGFR which leads to lysosome-mediated turnover, while pVHL promotes polyubiquitination on the activated EGFR which leads to proteasomal degradation.

and subsequent proteasomal degradation that is independent of c-Cbl (**Figure 2**). Further study is needed to determine the relative contributions of the HIF-dependent and HIF-independent mechanisms that pVHL uses to suppress activated EGFR. Nevertheless, in *VHL*-defective ccRCC cells, the prolonged signaling of the activated EGFR, together with elevated TGF-α and EGFR protein level, likely contributes to tumor growth. As the activated EGFR that is phosphorylated at some sites displayed VHL-dependent degradation (unpublished data), it is likely that pVHL-dependent polyubiquitination of activated EGFR is not a phenomenon that is unique to kidney cancer cells.

RNA POLYMERASE II SUBUNITS

Large subunit of RNA polymerase II (RPB1) is responsible for the initiation and elongation of mRNA and its activity is regulated through its c-terminal phosphorylation (Kuznetsova et al., 2003; **Table 1**). Through bioinformatic analysis, Kuznetsova et al. (2003) found that a fragment of RPB1 share some sequence similarity with oxygen-dependent degradation domain (ODDD) of HIF1α. In addition, RPB1 also contained an analogous LXXLAP sequence that was found in HIF1α protein that was the site of prolyl hydroxylation that mediated recognition by pVHL. In response to DNA damage agents or UV radiation, RPB1 underwent hyperphosphorylation and then proline hydroxylation. This led to recognition by pVHL-associated E3 ligase complex and its ubiquitination in PC12 cells. By testing the *in vitro* binding between RPB1 peptide and radio-labeled pVHL, they showed that hydroxylated RPB1 Proline 1465 was the major site that mediated interaction with pVHL. However, it remained to be determined whether hyperphosphorylation on RPB1 led to its proline hydroxylation. They also showed that the amount of hyperphosphorylated, but not hypophosphorylated RPB1 correlates inversely with pVHL levels in PC12 cells. These data suggests that pVHL binds to RPB1 through hydroxylated proline, promotes the ubiquitination of RPB1, and reduces hyperphosphorylated RPB1 levels in response to UV or DNA damage agents in PC12 cells.

Surprisingly, when pVHL was re-expressed in two different VHL-deficient kidney cancer cell lines, pVHL increased the level of RPB1 instead of decreasing it as expected (Mikhaylova et al., 2008). Furthermore, overexpression of wild type RPB1, not the P1465A mutant that cannot be hydroxylated, promoted tumor growth in kidney cancer cells expressing wild type VHL. Since the P1465A mutant RPB1 escapes VHL regulation, this is against the hypothesis that loss of VHL regulation on RPB1 is a tumor-promoting event. However, subsequent studies did reveal that RPB1 hydroxylation was significantly higher in kidney tumors compared to normal control. Consistent with the finding that PHD1 (EglN2, one of prolyl hydroxylases that modify HIFα) was the primary hydroxylase that modifies RPB1, levels of RPB1 hydroxylation correlated with levels of PHD1 in kidney cancer (Yi et al., 2010). Thus the contribution of RPB1 hydroxylation and pVHL-dependent ubiquitination to kidney cancer remains unclear and awaits further investigation.

In addition to RPB1, Na et al. discovered a novel pVHL-interacting protein, human RNA polymerase II seventh subunit (hsRPB7) by performing yeast two-hybrid screening from a kidney cDNA library. hsRBP7 bound to the 54–113 amino acid regions of pVHL, a part of VHL β-domain responsible for substrate recognition (Na et al., 2003). Interestingly, two representative VHL β-domain mutants (P86H and Y98H) showed decreased binding to hsRPB7 compared to wild type. hsRBP7 underwent VHL-dependent ubiquitination and proteasome-dependent degradation. As a functional readout, hsRPB7 can positively regulate VEGF expression, an effect that was ameliorated by overexpressing VHL in kidney cancer cells. However, in this paper, it was not clear whether hydroxylation would play a role in hsRBP7's degradation by pVHL E3 ligase complex. It will also be critical to identify the molecular mechanism by which hsRPB7 regulates the expression of specific genes such as VEGF.

PROTEIN KINASE C

Protein kinase C (PKC) is a superfamily of phospholipid-activated serine/threonine kinase. Activation of canonical PKC family members by 12-*O*-tetradecanoylphorbol-l3-acetate (TPA) can lead to various cellular responses, such as change of cell morphology and increased cell proliferation (Hata et al., 1993; **Table 1**). Various PKC family members were reported to bind to VHL, and this led to their ubiquitination and degradation. Although PKCδ was found to interact with the β domain of pVHL, its overall protein level was not affected by pVHL status (Iturrioz et al., 2006). Instead it was PKCζII that was reported to be a pVHL substrate, and its c-terminus was important for VHL-dependent proteasomal degradation (Iturrioz and Parker, 2007). While wild type pVHL could promote the degradation of PKCζII efficiently, some tumor-derived VHL mutants (such as Y98H, C162W, and R167W) failed to do so. However, it remained unclear whether PKCζII was a direct substrate for pVHL complex, and whether proline hydroxylation played a role in the turnover of PKCζII.

Protein kinase Cλ/4RA contains point mutations that render it partially and constitutively active (Akimoto et al., 1996). The active form of PKCλ bound to pVHL tighter than its wild type

Table 1 | A brief description of the pVHL substrates and the types of cancer they are involved in.

Gene name	Biological functions	Types of cancer involved	Reference
HIFα (HIF1, 2, and 3α)	Mediate transcriptional adaptation to oxygen deprivation by enhancing metabolic change, migration, and angiogenesis	All types of cancer	Semenza (2010)
EGFR (epidermal growth factor receptor)	Activate Ras/Raf/MEK/ERK and PI3K/PDK1/Akt1 pathways; promote cell proliferation; and resistance to apoptosis	All types of cancer	Jorissen et al. (2003), Yarden and Sliwkowski (2001)
RPB1 (large subunit of RNA polymerase II)	The largest subunit of RNA polymerase II, the polymerase responsible for synthesizing messenger RNA in eukaryotes	Kidney cancer	Yi et al. (2010)
RPB7 (RNA polymerase II seventh subunit)	The seventh largest subunit of RNA polymerase II that reportedly increases VEGF expression	Kidney cancer	Na et al. (2003)
aPKC (atypical protein kinase C)	Activates MAPK and upregulate VEGF expression (PKCδ); phosphorylates MUC1 and potentiates β-catenin signaling (PKCδ); increases cancer cell migration (PKCδ); acts as endogenous inhibitors of tight junction formation (PKCζII)	Breast cancer Colon cancer Kidney cancer Endometrial cancer	Pal et al. (1997), Ren et al. (2002), Razorenova et al. (2011), Reno et al. (2008), Parkinson et al. (2004)
SPRY2 (sprouty2)	Antagonizes the activated receptor tyrosine kinases and downregulates angiogenesis	Breast cancer Hepatocellular cancer prostate cancer Lung cancer Colon cancer	Lee et al. (2001), Lo et al. (2004), Fong et al. (2006), McKie et al. (2005), Sutterluty et al. (2007), Feng et al. (2011)
β2AR (β-adrenergic receptor II)	Mediate the catecholamine-induced activation of adenylate cyclase through the action of G proteins. involved in cardiovascular functions and apoptosis	Currently not known	Rockman et al. (2002)
MYBBP1A (Myb-binding protein p160)	May activate or repress transcription through interactions with DNA-binding proteins	Head and neck squamous cell carcinoma	Diaz et al. (2007), Tavner et al. (1998), Acuna Sanhueza et al. (2012)

counterpart, and this led to its preferential ubiquitination by VHL E3 ligase complex (Okuda et al., 2001).

Atypical PKCλ interacted with ASIP/PAR-3 and PAR-6 and was important for the maintenance of the tight junctions and the cell polarity in epithelial cells (Suzuki et al., 2001). In another epithelial cell line HC11 PKCζII was critical to maintain the cells in a non-differentiated state characterized by the absence of tight junctions and cell overgrowth (Parkinson et al., 2004). Since pVHL was reported to bind and degrade several PKC family members, it was reasonable to hypothesize that pVHL can affect actin and cytoskeletal organization, tight junction formation and cell polarity, which were often found dysregulated in cancer cells. Further study is needed to investigate the functional role of the VHL–PKCs axis in kidney cancer.

SPROUTY2

Sprouty2 (SPRY2) is one of four mammalian sprouty family members (SPRY1–4; Hacohen et al., 1998). Previous research showed that SPRY family members negatively regulated the activities of receptor tyrosine kinase and reduced angiogenesis (Lee et al., 2001; **Table 1**). Anderson et al. (2011) reported that hypoxia increased SPRY2 protein levels in various cancer cells, and it did so mainly through increased SPRY2 protein stability. While knockdown of PHD1 or PHD3 increased SPRY2 protein levels, overexpression of all three PHD isoforms (PHD1, 2, and 3) decreased its protein levels. By mass spectrometry, three potential prolyl hydroxylation sites were identified (P18, 144, and 160). Mutating these three Proline residues to Alanine residues significantly decreased the binding between SPRY2 and pVHL and produced more stable SPRY2 protein. Functionally, since SPRY2 was reported to have anti-migratory and anti-proliferative effect on cancer cell growth through inhibiting ERK1/2 kinase pathway (Impagnatiello et al., 2001; Yigzaw et al., 2001), suppressing either PHD1 or pVHL blunted the effect of FGF-induced ERK kinase pathway due to increased SPRY2 protein level. In a subset of hepatocellular carcinoma, pVHL protein levels were upregulated, and this led to decrease of SPRY2 protein that contributed to cancer progression (Lin et al., 2008). However, about 70% renal cell carcinomas have defects in pVHL. If the SPRY2 protein levels are upregulated in these tumors as expected, it will be intriguing to find out how this would impact tumorigenesis.

β_2-ADRENERGIC RECEPTOR

β_2-adrenergic receptor (β_2AR) is one of the G-protein-coupled receptors (GPCRs). Besides generating second messengers, β_2AR plays an important role in control of cardiovascular functions and apoptosis (Rockman et al., 2002; **Table 1**). Xie et al. (2009) reported that hypoxia can stabilize β_2AR protein by inhibiting its ubiquitination. Further findings demonstrated that pVHL E3 ligase complex associated with β_2AR protein *in vivo*, contributing to its ubiquitination and degradation. Mechanistically, prolyl

hydroxylase PHD3 interacted with β_2AR and mediated the hydroxylation of the β_2AR at proline residues 382 and 395, which primed β_2AR recognition by pVHL E3 ligase complex. β_2AR accounts for 25–30% of total β-type adrenergic receptor in the human heart and is the predominant form of the adrenergic receptor that exists in some of smooth muscles (Johnson, 1998; Rockman et al., 2002). Interestingly, the β_2AR protein is highly expressed in heart *in vivo*, where PHD3 is also abundantly expressed (Xie et al., 2009). This poses an apparent paradox, as PHD3 is the major enzyme that modifies β_2AR for its degradation. It might be possible that the PHD3-dependent destruction of β_2AR is a regulated event and only happens with external stimuli. Although it is unclear now, the PHD3–β_2AR–pVHL signaling axis might be operating in kidney cancer and merits more investigation.

Myb-BINDING PROTEIN p160 (MYBBP1A)

Using an ICAT (isotope-coded affinity tag) quantitative proteomics technology, Lai et al. identified MYBBP1A as a novel VHL substrate. MYBBP1A is a transcriptional regulator that can activate or suppress gene transcription through interacting with DNA-binding proteins (Tavner et al., 1998; Diaz et al., 2007; **Table 1**). MYBBP1A was degraded by VHL in a prolyl hydroxylation dependent manner. Further research showed that MYBBP1A proline 693 site as the potential hydroxylation site that might trigger the interaction with VHL and subsequent degradation (Lai et al., 2011). It remains largely unknown how MYBBP1A might contribute to kidney cancer.

SUMMARY

Proline hydroxylation on HIFα proteins led to their recognition by pVHL, followed by very efficient ubiquitination and proteasomal degradation. Without a functional VHL, HIF pathway is strongly and constitutively active. So far this has been proved to be the most significant and clinically useful tumor suppressor function of pVHL. However, although proline hydroxylation on RPB1, SPRY2, and β_2AR also led to pVHL-dependent ubiquitination, this did not automatically cause protein degradation as the ubiquitin chain linkages on them might be different. It is also unclear whether SPRY2 and β_2AR are more abundant in VHL-defective renal cancer cells and whether they contribute to tumor growth or maintenance at all.

Interestingly, it is the active forms of EGFR and atypical PKC that are ubiquitinated by pVHL and targeted for degradation. As these kinases have pro-proliferating and anti-survival activities, it is possible that their degradation is also important to pVHL's tumor suppressor function. It remains to be tested whether protein phosphorylations, in addition to proline hydroxylation, also constitute a recognition signal for pVHL.

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Pathogenic role of the CRL4 ubiquitin ligase in human disease

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The cullin 4-RING ubiquitin ligase (CRL4) family employs multiple DDB1–CUL4 associated factors substrate receptors to direct the degradation of proteins involved in a wide spectrum of cellular functions. Aberrant expression of the cullin 4A (*CUL4A*) gene is found in many tumor types, while mutations of the cullin 4B (*CUL4B*) gene are causally associated with human X-linked mental retardation. This focused review will summarize our current knowledge of the two CUL4 family members in the pathogenesis of human malignancy and neuronal disease, and discuss their potential as new targets for cancer prevention and therapeutic intervention.

Keywords: cullin, CUL4A, CUL4B, CRL, cancer, disease

INTRODUCTION

The cullin-RING ubiquitin ligases (CRLs) are the largest E3 ligase family in eukaryotes, and ubiquitinate a wide array of substrates involved in cell cycle, signaling, DNA damage response, gene expression, chromatin remodeling, and embryonic development. Cullins serve as elongated scaffolds that assemble functional E3 complexes by utilizing distinct adaptors to recruit substrate receptors and the ubiquitin-charged E2 conjugating enzyme (**Figure 1**). Within the E2–E3 complex, the RING domain-containing Rbx1/ROC1/Hrt1 or Rbx2/SAG adaptor bridges E2 binding to the cullin carboxyl terminus, which is necessary for transfer of ubiquitin to all cullin substrates. The cullin amino terminus binds cullin-specific adaptors, which in turn recruit distinct classes of substrate receptors that target substrates to the E2–CRL complex for ubiquitination and subsequent degradation by the 26S proteasome. CRL activity is regulated by the Nedd8 post-translational modification: the ubiquitin-like Nedd8 protein is conjugated to cullins in a manner highly analogous to ubiquitination. In a cascade of events designated the neddylation cycle, neddylation promotes CRL activity, while deneddylation by the COP9 signalosome inhibits cullins (reviewed in Petroski and Deshaies, 2005; Sarikas et al., 2011).

Among the eight cullins (CUL1-7 and PARC) in higher organisms, the CUL4 subfamily of CRLs is uniquely comprised of two members, CUL4A and CUL4B, that share extensive sequence homology and functional redundancy. While most cullins recruit substrate receptors using BTB domain-containing adaptors, the CUL4 family employs the structurally distinct triple WD40 β-propeller domain-containing DDB1 adaptor to recruit members of the DDB1–CUL4 associated factors (DCAF) family of substrate receptors (Angers et al., 2006; He et al., 2006; Higa et al., 2006a; Jin et al., 2006; reviewed in Lee and Zhou, 2007). Genetic approaches have been utilized to dissect the physiological relevance and unique functions of the CUL4 family members, as well as the individual components of the CRL4 ubiquitin ligase complex (Yoon et al., 2004; Kopanja et al., 2009, 2011; Liu et al., 2009; Yin et al., 2011). This focused review will summarize recent findings that have shed light on the role of CUL4 activity in human disease.

CUL4A AND CANCER

CUL4A was initially identified as an amplified or overexpressed gene in primary human breast cancers (Chen et al., 1998). Genome-wide analysis of human cancers revealed *CUL4A* amplification in 5% of familial and sporadic breast cancers, and as high as 20% in the basal-like breast cancer subtype that is associated with aggressive growth and poor prognosis (Melchor et al., 2009). *CUL4A* amplification has also been found in squamous cell carcinomas (Shinomiya et al., 1999), adrenocortical carcinomas (Dohna et al., 2000), childhood medulloblastoma (Michiels et al., 2002), hepatocellular carcinomas (Yasui et al., 2002), and primary malignant pleural mesotheliomas (Hung et al., 2011). Recently, genome-wide high-density SNP arrays further revealed high *CUL4A* gene copy number in a subset of lung and ovarian carcinomas, as well as other solid tumor types (Beroukhim et al., 2010). Moreover, high CUL4A expression correlates with significantly shorter overall and disease-free survival (Schindl et al., 2007), indicating that dysregulation of CUL4A may play a role in promoting oncogenesis. Mouse models support this hypothesis, as skin-specific *Cul4a* knockout mice showed marked resistance to UV-induced carcinogenesis compared to wild-type and heterozygous mice (Liu et al., 2009). Transgenic mice with inducible expression of exogenous CUL4A developed pulmonary hyperplasia, which is consistent with a role for dysregulated CUL4A in driving uncontrolled proliferation (Li et al., 2011a). The role of CUL4B in carcinogenesis remains to be determined.

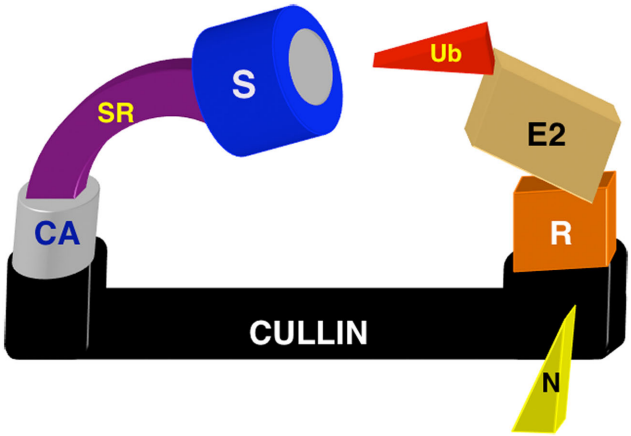

FIGURE 1 | Schematic diagram of the multimeric CRL ubiquitin ligase complex. The elongated cullin scaffold binds cullin-specific adaptors (CA) at the N-terminus to recruit substrates (S). All cullins except CUL3 require separate substrate receptors (SR) to bridge the adaptor–substrate interaction. RING protein adaptors (R) bind the ubiquitin (Ub)-charged E2 conjugating enzyme at the cullin C-terminus. Nedd8 post-translational modification (N) promotes cullin activity.

The damaged DNA binding proteins DDB1 and DDB2 were first characterized as DNA damage sensors that initiate the nucleotide excision repair (NER) pathway following UV irradiation (reviewed in Tang and Chu, 2002). Earlier studies identified the DDB1–DDB2 heterodimer as both a target and component of the CRL4 ubiquitin ligase complex (Shiyanov et al., 1999; Chen et al., 2001; Nag et al., 2001; Groisman et al., 2003). *DDB2* mutations that impair the recognition of UV-induced DNA lesions are causal for the photosensitivity and early onset of skin cancer found in xeroderma pigmentosum group E (XPE) patients (Nichols et al., 2000), and were recapitulated in the *Ddb2* knockout mouse model (Itoh et al., 2004; Yoon et al., 2004; Alekseev et al., 2005). Conversely, enforced expression of *DDB2* in transgenic mice delayed the onset of UV-induced squamous cell carcinomas (Alekseev et al., 2005), further highlighting the significance of DDB2 activity in DNA repair and cancer prevention. The physiological relevance of CUL4A-mediated degradation of DDB2 was determined in the *Cul4a* knockout mouse, as skin-specific deletion of CUL4A significantly enhanced resistance to UV-induced skin carcinogenesis (Liu et al., 2009). Protein levels of DDB2 and XPC, another NER damage sensor and $CRL4^{DDB2}$ substrate (Sugasawa et al., 2005), were found to accumulate, thus augmenting NER activity and decreasing tumorigenic potential.

In addition to DNA repair, CRL4 also plays a significant role in cell cycle regulation by targeting the Cdt1 DNA replication licensing factor, the p21 cyclin-dependent kinase inhibitor, and the PR-Set7/Set8 histone H4K20 methyltransferase for ubiquitin-proteolytic degradation in a Cdt2 (DCAF)- and PCNA-dependent manner (Higa et al., 2003; Zhong et al., 2003; Hu et al., 2004; Jin et al., 2006; Nishitani et al., 2006, 2008; Abbas et al., 2008, 2010; Kim et al., 2008; Centore et al., 2010; Oda et al., 2010; Tardat et al., 2010; Jorgensen et al., 2011). Knockdown of Cdt2 resulted in G2 arrest and DNA re-replication of the genome (Jin et al., 2006), indicating a critical role for $CRL4^{Cdt2}$ in limiting the replication of DNA during S phase. In response to UV or ionizing radiation, Cdt1, p21, and PR-Set7/Set8 were rapidly degraded in a CRL4-dependent manner (Higa et al., 2003; Hu et al., 2004; Abbas et al., 2008, 2010; Centore et al., 2010; Jorgensen et al., 2011). S phase arrest is also triggered by CRL4-mediated degradation of Chk1 in a phosphorylation-dependent manner under normal conditions and in the presence of genotoxic stress (Zhang et al., 2005; Leung-Pineda et al., 2009). While these proteins are targeted by both CUL4 family members, p21 protein levels were found to accumulate in primary $Cul4a^{-/-}$ mouse embryonic fibroblasts (MEFs) following UV irradiation, resulting in prolonged G1/S arrest (Liu et al., 2009). Higher p21 levels enforced the G1/S cell cycle checkpoint post-UV, thus allowing additional time for NER activities to conclude prior to the initiation of DNA replication. The absence of G2 arrest or DNA re-replication in the *Cul4a* knockout mouse model indicates that CUL4B at least partially compensates for the loss of CUL4A activity. Simultaneous inactivation of both CUL4A and CUL4B in primary MEFs led to growth arrest (Liu et al., 2009), which recapitulates the rapid G1 arrest observed in DDB1 knockout MEF cells (Cang et al., 2006).

Conflicting reports indicate that additional cell cycle regulators may be targeted by CRL4-based ubiquitin ligases for proteasome-mediated degradation. CUL4A overexpression reduced the steady-state levels of the CDK inhibitor $p27^{Kip1}$ in 293T cells, while CUL4A shRNA or dominant-negative CUL4A resulted in the accumulation of $p27^{Kip1}$ in mouse mammary epithelial cells or the human MCF-7 breast cancer cell, respectively (Miranda-Carboni et al., 2008). However, the turnover rate of $p27^{Kip1}$ was not directly measured under these conditions. Using primary MEF cells, Cang et al. (2006) showed that deletion of DDB1 resulted in G1/S cell cycle arrest and $p27^{Kip1}$ accumulation. However, the half-life of p27 was not prolonged in the absence of DDB1, arguing against $p27^{Kip1}$ as a direct substrate of the CRL4 ubiquitin ligase. Future studies should determine whether a CRL4-based E3 ligase directly or indirectly regulates $p27^{Kip1}$ protein stability. Interestingly, silencing of CUL4B in primary MEF cells had little effect on cell proliferation (Liu et al., 2009), but CUL4B knockdown in HeLa cells resulted in S phase cell cycle arrest (Zou et al., 2007) as well as cyclin E accumulation (Higa et al., 2006b; Zou et al., 2009). Given the stimulatory role of cyclin E in S phase progression and cell proliferation, it remains to be determined how increased cyclin E levels would result in growth inhibition, and whether additional CUL4B substrates are also involved in triggering S phase arrest.

The amplification or overexpression of *CUL4A* observed in various cancers likely corresponds with diminished post-translational stability of its substrates, many of which are tumor suppressors (**Table 1**). $CRL4^{Fbw5}$ may promote oncogenesis by targeting the mTOR inhibitor tuberous sclerosis protein 2 (Tsc2) for ubiquitination (Hu et al., 2008). Mutations in the Tsc1 and Tsc2 tumor suppressors are causal for the synonymous autosomal dominant disease that is marked by the formation of benign growths on the skin, nervous system, kidneys, and heart. REDD1, another inhibitor of mTOR signaling, is targeted for degradation by the $CRL4^{\beta TrCP}$ ubiquitin ligase (Katiyar et al., 2009). Additional tumor suppressors that are subject to CUL4A-mediated ubiquitination include p150/Sal2, which is degraded as cells transition from quiescence to

Table 1 | Involvement of CUL4A substrates in pathogenesis.

DCAF	Substrate	Substrate functions	Associated pathogenesis	Reference
DDB2	DDB2	Nucleotide excision repair; DCAF substrate receptor.	Xeroderma pigmentosum; skin cancer	Nichols et al. (2000), Chen et al. (2001), Groisman et al. (2003), Nag et al. (2001), Sugasawa et al. (2005)
DDB2	XPC	Nucleotide excision repair	Xeroderma pigmentosum, skin cancer	Sugasawa et al. (2005)
Cdt2	p21/CIP/WAF1	CDK inhibitor	Normal cell cycle and DNA damage response	Abbas et al. (2008), Kim et al. (2008), Nishitani et al. (2008)
Cdt2	Cdt1	DNA replication licensing factor	DNA re-replication	Higa et al. (2006a), Jin et al. (2006)
Cdt2	PR-Set7/Set8	Histone methyltransferase	Unknown	Abbas et al. (2010), Centore et al. (2010), Jorgensen et al. (2011), Oda et al. (2010), Tardat et al. (2010)
Fbw5	Tsc2	Inhibitor of mTOR signaling	Tuberous sclerosis	Hu et al. (2008)
β-TrCP	REDD1	Inhibitor of mTOR signaling	unknown	Katiyar et al. (2009)
RBBP7	p150/Sal2	Inhibitor of cell growth	Putative tumor suppressor	Sung et al. (2011)
	RASSF1A	Inhibitor of Ras signaling	Putative tumor suppressor	Jiang et al. (2011)
TRCP4AP/TRUSS	N-Myc, C-Myc	Transcription factors	Oncoproteins, multiple tumors	Choi et al. (2010)
COP1	c-Jun	Transcription factor	Oncoprotein, multiple tumors	Wertz et al. (2004)
COP1	p53	Transcription factor	Tumor suppressor	Dornan et al. (2004)
COP1	ETV1	Transcription factor	Oncoprotein, prostate cancer	Vitari et al. (2011)
DCAF1/VprBP	unknown	Cell cycle regulator	Oncoprotein. Inhibited by the Merlin tumor suppressor	Li et al. (2010)
Cereblon	unknown	Limb development/patterning	Terotogenic, multiple myeloma. Inhibited by thalidomide	Ito et al. (2010)
Unknown	HOXA9	Transcription factor	Acute myeloid leukemia	Zhang et al. (2003)
Unknown	p27/Kip1	CDK inhibitor	Tumor suppressor	Higa et al. (2006b)
Unknown	cyclin E	Cell cycle progression	Oncoprotein, multiple tumors	Higa et al. (2006b)
Unknown	Chk1	Cell cycle checkpoint kinase	Tumor suppressor	Zhang et al. (2005), Leung-Pineda et al. (2009), Guervilly et al. (2011)

an actively dividing state (Sung et al., 2011), and RASSF1A (Jiang et al., 2011), a negative Ras effector that was previously identified as a target of the CRL1/SCF^{Skp2} (Skp1, CUL1, F-box-containing substrate receptor, and Rbx1) ubiquitin ligase (Song et al., 2008). Contrary to the trend of CUL4A-mediated degradation of tumor suppressors, the CRL4 substrate receptor TRCP4AP/TRUSS targets both N-Myc and C-Myc transcription factors for degradation (Choi et al., 2010). Stabilization of Myc protein in many cancer cell lines corresponds with TRCP4AP/TRUSS downregulation, indicating the potential significance of CUL4-mediated post-translational regulation in restricting Myc protein levels. COP1, acting as a CRL4 substrate receptor, functions as a tumor suppressor by targeting the c-Jun proto-oncogene for ubiquitination (Wertz et al., 2004). However, tissue-specific differences have been reported in CRL4-independent COP1 ubiquitin ligase activity. The p53 tumor suppressor is targeted for degradation by COP1 (Dornan et al., 2004), which may provide mechanistic insight into COP1 overexpression observed in ovarian and breast cancer. Conversely, the oncogenic ETS transcription factors are also degraded in a COP1-dependent manner, and loss of COP1 activity results in ETV1 accumulation that promotes prostate epithelial cell proliferation (Vitari et al., 2011). Finally, ectopic expression of *CUL4A* in PC12 rat pheochromocytoma cells suppresses apoptosis and promotes cell survival (Tan et al., 2011), further indicating that CUL4A activity supports cell growth.

The oncogenic effects of CUL4A are highlighted by the finding that Merlin, a tumor suppressor encoded by *NF2*, inhibits the ubiquitin ligase activity of CUL4A in complex with VprBP/DCAF1 (Li et al., 2010). Moreover, mutations in Merlin that ablate the enzymatic inhibition of CRL4^{VprBP} have been found in patients with the neurofibromatosis type 2 familial cancer syndrome (Li et al., 2010). VprBP silencing compromises tumorigenesis in Merlin-deficient mesothelioma cell lines, indicating the significance of CRL4^{VprBP} signaling in promoting cellular proliferation (Li et al., 2010). Huang and Chen showed that Merlin was targeted for degradation by the CRL4^{VprBP} ubiquitin ligase (Huang and Chen, 2008). However, Li et al. demonstrated that Merlin was not a substrate of CRL4^{VprBP}, but rather served as a negative regulator of the CRL4^{VprBP} ubiquitin ligase. Further biochemical and genetic studies are required to determine the functional relationship between Merlin and CRL4^{VprBP}. VprBP is also required for cell cycle progression into S phase (Belzile et al., 2007; Hrecka et al., 2007; Le Rouzic et al., 2007; Tan et al., 2007; Wen et al., 2007), but the mechanism of cell cycle regulation and the cellular targets of CRL4^{VprBP}

have yet to be determined. Nevertheless, these findings indicate a role for the $CRL4^{VprBP}$ ubiquitin ligase in promoting cell cycle progression and oncogenic transformation.

Despite the numerous growth-promoting pathways that are amplified by CUL4A activity, the degradation of other identified CUL4A substrates reveals a more complex effect on growth regulation. CUL4A plays a critical role in granulopoiesis by degrading the HOXA9 homeodomain protein, which may also restrict HOXA9-induced leukemogenesis (Zhang et al., 2003). The leukemogenic NUP98–HOXA9 fusion protein, which is derived from the *t*(9;11)(p15;p15) chromosomal translocation in acute myeloid leukemia patients, is resistant to CUL4A-mediated degradation, further indicating that CUL4A may play a role in the proper differentiation of hematopoietic cells (Chung et al., 2006). In addition to restricting the NER threshold, CRL4 also responds to DNA damage through histone H3 and H4 ubiquitination, which facilitates the recruitment of repair proteins (e.g., XPC) to damaged DNA (Wang et al., 2006). Thus, the CUL4A ubiquitin ligase may play distinct roles in tumorigenesis that are dictated by cellular context or environmental conditions.

CUL4B AND HUMAN X-LINKED MENTAL RETARDATION

CUL4B mutations in human patients have been found to be causal for X-linked mental retardation (XLMR) syndrome (Tarpey et al., 2007; Zou et al., 2007; Badura-Stronka et al., 2010; Isidor et al., 2010). Patient-derived cells also display increased camptothecin-induced topoisomerase I-dependent DNA breaks, which are associated with the peripheral neuropathy spinocerebellar ataxia with axonal neuropathy-1 (SCAN-1; Kerzendorfer et al., 2010). The unique CUL4B N-terminus may mediate the recruitment of distinct substrates for degradation, and their accumulation likely contributes to the CUL4B phenotype. WDR5, a subunit of the H3K4 methyltransferase complex, was initially identified as a DCAF and more recently characterized as a CUL4B substrate (Nakagawa and Xiong, 2011). XLMR-derived *CUL4B* mutations resulted in the accumulation of WDR5 and subsequent activation of neuronal genes that promote neurite extension. Peroxiredoxin III is another unique CUL4B substrate that may affect neural development through the regulation of reactive oxygen species (ROS) levels (Li et al., 2011b). Finally, the arylhydrocarbon receptor (AhR) acts as a unique CUL4B substrate receptor that targets estrogen and androgen receptors for proteasome-mediated degradation (Ohtake et al., 2007), further highlighting the role of CUL4B as a transcriptional regulator.

Distinct expression patterns may also account for the phenotypes observed in the *Cul4a* knockout mouse model. In addition to enhanced resistance against UV-induced skin carcinogenesis, CUL4A knockout also resulted in male infertility (Kopanja et al., 2011; Yin et al., 2011). These studies revealed non-overlapping expression patterns between CUL4A and CUL4B during the pachytene to diplotene stages in adult testes, thus accounting for the physiological requirement for CUL4A activity in spermatogenesis (Yin et al., 2011). The disease syndrome manifested in humans with CUL4B mutations may be attributed to exclusive substrate targeting and/or differential expression patterns of the CUL4 family members. *In vivo* interrogation of CUL4B activity using knockout mouse models would shed insight into the mechanism of pathology, and may identify avenues for therapeutic intervention.

CRL4 AS A CANDIDATE FOR THERAPEUTIC INTERVENTION

Manipulation of the post-translational stability of cellular proteins has been demonstrated to be a new and effective cancer therapeutic strategy. Proteasome inhibitors, such as bortezomib, non-specifically block the overall degradation of poly-ubiquitinated proteins, but preferentially sensitize rapidly dividing cells to apoptosis (Richardson et al., 2003; Kane et al., 2007; Orlowski and Kuhn, 2008). MLN4924, a small molecule inhibitor of the Nedd8 E1 activating enzyme, specifically blocks cullin activity, which leads to the accumulation of their substrates (Soucy et al., 2009). However, more precise targeting of CRL complexes has been shown to be possible with the discovery that the teratogenic agent thalidomide specifically inhibits Cereblon (CRBN) (Ito et al., 2010), an identified DCAF for the CRL4 ubiquitin ligase family. Substrates for $CRL4^{CRBN}$ have yet to be determined, but aberrant Fgf8 expression and signaling were observed following Cereblon inhibition (Ito et al., 2010). Thalidomide is currently used to treat multiple myeloma, indicating that Cereblon activity likely promotes cell growth.

Viral hijack of CRL4 activity may provide further insight into possible mechanisms of CRL4 intervention. Parainfluenza virus 5 (PIV5, formerly SV5) V protein binds DDB1, which recruits STAT2 to target STAT1 for ubiquitination and proteasome-mediated degradation, thus inhibiting the cellular interferon-induced response to viral infection (Precious et al., 2005, 2007). Additional rubulaviruses, such as human parainfluenza virus 2 and mumps virus, also target STAT proteins for degradation by redirecting DDB1 activity through their V proteins (Ulane and Horvath, 2002). DDB1 binding by the hepatitis B virus X protein (HBx) resulted in S phase arrest (Martin-Lluesma et al., 2008), and the subsequent deleterious effects contributed to hepatocellular carcinomas in a cell-non-autonomous manner that was recapitulated in hepatocyte-specific *Ddb1* knockout mice (Yamaji et al., 2010). S phase arrest is also triggered by HIV-1 Vpr and HIV-2 Vpx proteins through $CRL4^{VprBP}$ binding, which may promote macrophage infection (Sharifi et al., 2012). Thus, the modification of DDB1 substrate binding represents another method of altering CRL4 activity for therapeutic purposes.

CONCLUDING REMARKS

Despite the significant sequence conservation and functional redundancy of the CUL4 family members, CUL4A and CUL4B play strikingly diverse roles in human disease. CUL4A activity promotes oncogenesis, as demonstrated by the overexpression and/or amplification of CUL4A in several tumor types and the resistance to UV-induced skin carcinogenesis in the *Cul4a* knockout mouse model, while loss-of-function mutations in CUL4B are causal for human X-linked mental retardation syndrome. Surprisingly, gene-trapped inactivation of *Cul4b* in mice resulted in embryonic lethality (Cox et al., 2010). The genetic interrogation of CUL4B awaits the generation of a conditional *Cul4b* knockout mouse model to identify the unique *in vivo* contributions of CUL4B activity, and the major substrates that are responsible for CUL4B-dependent disease phenotypes. Furthermore, additional mouse

models are required to gain a comprehensive understanding of the role of CRL4 in pathogenesis through the functional delineation of the DCAF substrate receptors, their substrates (Emanuele et al., 2011), and the associated cellular pathways whose dysregulation contribute to pathogenesis.

CUL4A represents an ideal target for therapeutic intervention, as genetic ablation in mice resulted in a marked resistance to carcinogenesis. Additionally, the recent structural determination of CRL4 assembly and activation provides a molecular platform for the design of inhibitors (Angers et al., 2006; Li et al., 2006; Fischer et al., 2011). Although the extensive protein–protein interface between CUL4 and DDB1 may present a formidable challenge for direct intervention by small molecule inhibitors, allosteric inhibitors are an attractive alternative to attenuate CRL4 activity, especially given the stringent requirements of proximity and E2–E3 orientation for effective ubiquitin transfer. Finally, the striking efficacy of thalidomide in treating multiple myeloma and its inhibition of Cereblon activity indicate that developing specific inhibitors for multiple subunits or interfaces, e.g., DCAF binding to either CRL4 or substrates, may prove to be feasible therapeutic strategies.

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Coordinate to guard: crosstalk of phosphorylation, sumoylation, and ubiquitylation in DNA damage response

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Small ubiquitin-like modifier-1/2/3 (SUMO-1/2/3) and ubiquitin share similar structure and utilize analogous machinery for protein lysine conjugation. Although sumoylation and ubiquitylation have distinct functions, they are often tightly associated with each other to fine-tune protein fate in transducing signals to regulate a wide variety of cellular functions, including DNA damage response, cell proliferation, DNA replication, embryonic development, and cell differentiation. In this *Perspective*, we specifically highlight the role of sumoylation and ubiquitylation in ataxia-telangiectasia mutated (ATM) signaling in response to DNA double-strand breaks and hypothesize that ATM-induced phosphorylation is a unique node in regulating SUMO-targeted ubiquitylation in mammalian cells to combat DNA damage and to maintain genome integrity. A potential role for the coordination of three types of post-translational modification in dictating the tempo and extent of cellular response to genotoxic stress is speculated.

Keywords: ATM, phosphorylation, sumoylation, ubiquitylation, DNA damage response

Small ubiquitin-like modifier (SUMO) and ubiquitin (Ub) can be post-translationally attached to their target proteins. They share less than 20% identity of amino acid sequence; however, their structure and biochemical machinery catalyzing the modification are similar. Sumoylation and ubiquitylation are known to function distinctly in operating the fate of protein. Sumoylation is to conduct signals for nuclear translocation, transcriptional repression and protein recruitment, whereas lysine (Lys)-48-linked polyubiquitylation is a common mark for protein degradation in proteasome; Lys-63 linkage of Ub directs protein trafficking. Both SUMO and Ub participate in regulating a wide range of cellular functions, including DNA damage response (DDR), cell growth, differentiation, and development (Gill, 2004; Denuc and Marfany, 2010; Tang and Greenberg, 2010; Praefcke et al., 2011). Here, we specifically focus on the role of sumoylation and ubiquitylation in DDR.

DNA damage response is an evolved mechanism for cells to confront DNA lesions generated by endogenous or environmental agents. The cells sense DNA lesions, such as DNA double-strand breaks (DSB), mismatches, and bulky adducts to subsequently induce signaling pathways promoting cell cycle arrest, DNA repair, apoptosis, transcription, and chromatin remodeling (Jackson and Bartek, 2009). A large protein kinase family, phosphoinositide 3 kinase-like protein kinases (PIKKs) including ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia, and Rad3 related (ATR), and DNA-dependent protein kinase catalytic subunit (DNA-PKcs), participates in DDR by phosphorylating a subset of target proteins to regulate cellular functions and maintain genome integrity. Defective DDR causes the accumulation of DNA lesions and eventually leads to genome instability, a hallmark of cancer. Particularly, ATM plays a primary role in transducing DSB signal to a broad spectrum of proteins involved in DNA repair, cell cycle checkpoints, and apoptosis (Shiloh, 2003). Mutations at the *ATM* locus thereby elicit phenotypes reflective of defects in cell cycle regulation and apoptosis. Ataxia telangiectasia (A-T) patients are extremely sensitive to ionizing radiation, such as X- and γ-rays (Lavin, 2008). A-T cells do not exhibit cell cycle arrest at regular checkpoints when stressed by irradiation (Morrison et al., 2000). Distinct mutations of the *ATM* gene also predispose some patients to cancer, particularly lymphoma cancers in 30% of A-T patients and leukemia (Hunter, 2007; Lavin, 2008). Therefore, studies of ATM-regulated pathways in conjunction with DDR are crucial to understanding maintenance of genome integrity. Particularly, in this *Perspective*, we review multiple types of post-translational modification (PTM): phosphorylation, sumoylation, ubiquitylation, and their combinatorial effects in ATM signaling pathways.

ATM-REGULATED SUMOYLATION IN DDR

Sumoylation regulates ATM-mediated DNA damage signaling in both transcription and chromatin remodeling. A unique example that well-illustrates the relationship between ATM-induced phosphorylation and sumoylation is Krüppel-associated box (KRAB)-associated protein 1 (KAP1; also known as TRIM28 and TIF1β). KAP1 is a transcriptional co-repressor primarily responding to DNA damage and regulating cellular functions such as checkpoint control and apoptosis (Lee et al., 2007; Li et al., 2007). KAP1 has six putative sumoylation sites and its sumoylation at Lys-779 and Lys-804 are required for the interaction with chromatin remodelers including heterochromatin protein 1(HP1), SET domain, bifurcated 1 (SETDB1), nucleosome remodeling deacetylase (NuRD), and histone deacetylases (HDACs) to establish a silent state of

chromatin and to repress transcription (Ryan et al., 1999; Schultz et al., 2001, 2002; Sripathy et al., 2006; Ivanov et al., 2007; Lee et al., 2007). When cells are exposed to genotoxic stress, DSBs activate ATM to phosphorylate KAP1 at serine (Ser)-824 and result in a decrease of SUMO–KAP1 population, leading to the de-repression of KAP1-regulated DNA damage-responsive genes: *p21*, *GADD45α*, *BAX*, *NOXA*, and *PUMA* (White et al., 2006; Lee et al., 2007; Li et al., 2007). Furthermore, phosphorylation/sumoylation switch on KAP1 has been illuminated through research in protein phosphatases 1α and 1β (PP1α and PP1β). While PP1α directly interacts with KAP1 under basal condition, PP1β interacts with KAP1 only in response to genotoxic stress. Changes in the abundance of PP1α and PP1β have differential effects on phosphorylation and sumoylation of KAP1 under both unstressed and stressed conditions (Li et al., 2010). Although the underlying mechanism of phosphorylation-induced decrease of SUMO–KAP1 is still not clear, possibilities might include that ATM-induced phosphorylation activates SUMO proteases to de-sumoylate KAP1 or that ATM triggers the degradation of SUMO–KAP1.

Besides of transcriptional regulation, KAP1 also participates in chromatin configuration. Chromodomain helicase DNA binding protein 3 (CHD3; also known as Mi2α) interacts with SUMO–KAP1 through its SUMO-interacting motif (SIM). The binding of KAP1 and CHD3 configures KAP1-associated chromatin into a compact structure. Upon DNA damage, ATM-induced phosphorylation at Ser-824 of KAP1 interrupts the SUMO:SIM interaction between KAP1 and CHD3, leading to the relaxation of chromatin which is more accessible for DNA repair modules to come in to the damage sites and repair the lesions (Goodarzi et al., 2011). Although very few reports demonstrate the connection between ATM signaling and protein sumoylation, in the KAP1 case, the control of phosphorylation/sumoylation switch on KAP1 seems to play an indispensable role in transducing ATM signal. The switch on KAP1 has to be tightly regulated in order to respond to DNA damage and return to the basal state in a timely and proper way.

ATM-REGULATED UBIQUITYLATION IN DDR

Ubiquitylation serves as a critical signal for recruiting DNA repair machinery to the damage sites. Upon DNA damage, MRE11–Rad50–NBS1 (MRN) complex recognizes and processes the end of DSBs (Uziel et al., 2003). ATM is then recruited to phosphorylate H2AX at Ser-139, creating a docking site for the mediator of DNA damage checkpoint protein 1 (MDC1) to be recruited to the DNA damage foci. ATM further phosphorylates MDC1 in order to recruit ring finger protein 8 (RNF8)–HERC2–UBC13 complex to the foci to catalyze Lys-63 polyubiquitylation of H2A (Lou et al., 2006; Huen et al., 2007; Kolas et al., 2007; Bekker-Jensen et al., 2010). RNF168 is subsequently recruited to the Lys-63 polyubiquitylated H2A and amplifies Lys-63-linked poly Ub chains on H2A and H2AX around the foci (Doil et al., 2009). Meanwhile, RNF8 also catalyzes Lys-48 polyubiquitylation on the client proteins at damage sites. The Lys-48 polyubiquitylated substrates will then be turned over by recruiting p97-UFD1–NPL4 Ub-selective segregase (Meerang et al., 2011). The coordination of these signals ultimately recruits BRCA1–RAP80 complex, 53BP1, and Rad51 to facilitate DSB repair and checkpoint arrest (Kolas et al., 2007; Doil et al., 2009; Bekker-Jensen et al., 2010; Meerang et al., 2011). After repairing the damage, the removal of H2AX from the damage sites involves an acetylation-dependent ubiquitylation catalyzed by TIP60–UBC13 complex (Ikura et al., 2007).

Other than transducing signals to recruit repair proteins, ATM also represses transcription in *cis* to DSBs by establishing monoubiquitylation of H2A to inhibit RNA polymerase II function. Since monoubiquitylation at Lys-119 of H2A (uH2A) is associated with transcriptional repression, ATM-mediated transcriptional silencing was explored in tandem with uH2A. By inhibiting ATM, uH2A levels at DSBs are significantly decreased although the Lys-63-linked poly Ub chains and RAP80 levels around the foci are less affected. ATM therefore plays a critical role in maintenance of uH2A at DSBs through RNF8/RNF168, while the Lys-63-linked poly Ub chains serve as separate docking sites for recruitment of repair proteins such as BRCA1 complex. Furthermore, ATM-dependent uH2A stalls RNA polymerase II-mediated transcription in *cis* to the damage site. A deubiquitylation enzyme, USP16, negatively regulates uH2A-dependent function and rapidly restores transcription after the cessation of DNA damage (Shanbhag et al., 2010).

Ataxia-telangiectasia mutated-induced phosphorylation also exhibits crosstalk with ubiquitylation by mediating protein degradation through direct and indirect recruitment of Ub E3 ligases. In the case of the tumor suppressor p53, ATM-induced phosphorylation participates in the dual function of repressing and promoting the ubiquitylation of different effectors, leading to their degradation. p53 induces transcription of multiple genes important for cell cycle regulation, DNA repair, and apoptosis. ATM phosphorylates p53 at Ser-15, leading to transcription of the CDK2/cyclin-E inhibitor which functions at the G1-S checkpoint (Shiloh, 2003). ATM-induced phosphorylation inhibits negative regulators of p53, including MDM2 and constitutive morphogenic 1 (COP1). Both MDM2 and COP1 are Ub E3 ligases that ubiquitylate p53 to promote its proteasomal degradation. ATM indirectly regulates MDM2-mediated degradation of p53 through phosphorylation of Chk2 which then phosphorylates p53 at Ser-20 to prevent the formation of the MDM2–p53 complex (Dumaz et al., 2001). ATM also directly phosphorylates MDM2 at Ser-395 to prevent the export of the MDM2–p53 complex into the cytoplasm, thereby maintaining p53 in the nucleus (Maya et al., 2001; Chen et al., 2005). In addition, phosphorylation of COP1 by ATM induces autoubiquitylation of COP1 (Dornan et al., 2006). ATM phosphorylation thus selectively influences the repression and activation of ubiquitylation on different proteins in response to DNA damage. Taken together, ATM-induced phosphorylation in coordination with ubiquitylation plays an essential role in establishing a series of signals directing to transcriptional regulation, the completion of DSB repair and in determining the fate of key proteins involved in DDR.

CROSSTALK BETWEEN SUMOYLATION AND UBIQUITYLATION IN DDR

The convergence of sumoylation and ubiquitylation does take place under genotoxic condition. When DSBs occur, protein inhibitor of activated signal transducer and activator of transcription (PIAS) localizes to the damage sites. PIAS1 and PIAS4 function as SUMO E3 ligase to modify BRCA1, 53BP1 and possibly

RNF8, RNF168 to modulate their activities in facilitating Ub signal amplification and DNA repair after genotoxic stress (Galanty et al., 2009). Sumoylation on BRCA1 increases its Ub E3 ligase activity, therefore termed as a SUMO-regulated Ub ligase (SRUbL; Morris et al., 2009). It is still not clear how PIAS activity is regulated under DNA damage condition, and whether this is dependent on ATM-induced phosphorylation. PIAS1 is phosphorylated by IκB kinase alpha (IKKα; Liu et al., 2007), whose activity is also regulated by ATM-dependent NF-κB essential modulator (NEMO) ubiquitylation (Wuerzberger-Davis et al., 2006), implying that the role of PIAS in DDR might also be incorporated into ATM signaling.

Another interesting example showing the interplay between sumoylation and ubiquitylation in the context of genotoxic stress is SUMO-targeted ubiquitin ligase (STUbL), including Slx8–Rfp, MIP1, Slx5–Slx8, and RNF4. It is a new class of ubiquitin E3 ligases targeting sumoylated proteins through their SIMs for ubiquitylation. STUbL has been reported to trigger the degradation of sumoylated proteins and is responsible for the maintenance of cell survival and genome stability (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008; Cook et al., 2009; Heideker et al., 2009). For example, RNF4 (RING finger protein 4, RING: Really Interesting New Gene), the only human homolog of Slx8–Rfp, is involved in arsenic-trioxide (ATO)-induced polyubiquitylation and proteasomal degradation of promyelocytic leukemia (PML) by targeting the poly SUMO-2 chain on PML (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). This reveals the role of RNF4 in mediating the crosstalk between sumoylation and ubiquitylation and also provides a possible mechanism of ATO-induced damage response. In conclusion, crosstalk between sumoylation and ubiquitylation seems like a general scenario in DDR; however, it is still not fully understood how this crosstalk might be modulated and since ATM signaling requires sumoylation and ubiquitylation to respond to DSB, it is possible that ATM is involved in tuning SUMO–Ub crosstalk through RNF4 in DDR.

IS RNF4 INVOLVED IN ATM SIGNALING?

Given to the fact that RNF4 biologically functions as an important factor for maintaining genome integrity and its ability to recognize and possibly regulate more than 300 substrates involved in a wide variety of biological processes, including chromatin remodeling and DNA repair etc. Bruderer et al. (2011), one could speculate that RNF4 might participate in ATM-regulated DSB damage response.

RNF4 IS EXTENSIVELY INVOLVED IN DNA DAMAGE SIGNALING PATHWAYS

Yeast homolog of RNF4, Slx5/Slx8 physically associates with DSBs to form damage foci, in a SUMO and SIM-dependent way (Nagai et al., 2008; Cook et al., 2009). Slx8 functions with Rad60, a DNA repair protein, and Nse2, a SUMO ligase to protect the genome from Topoisomerase-1 (Top-1)-induced DNA damage (Prudden

FIGURE 1 | Sequence alignment of SIMs and ARR of RNF4 family members from different organisms. Data here show that four SIM domains and ARR are conserved throughout evolution.

et al., 2007; Heideker et al., 2011). Fission yeast Rfp1 and Rfp2 complement one another in regulating defects in cell cycle progression and Chk1-dependnet DNA repair; moreover, human RNF4 is able to functionally rescue this phenotype in *rfp1/rfp2* double null mutant (Kosoy et al., 2007). RNF4 also functions specifically to demethylate DNA by interacting with base excision repair enzymes TDG and APE1 that target G:T mismatches in the DNA. In addition, RNF4 deficiency displays global DNA hypermethylation (Hu et al., 2010). Taken together, the biological function of RNF4 is conserved and tightly associated with DDR, especially DNA repair and chromatin remodeling in different organisms, supporting the idea that RNF4 plays a role in coordinating and transducing ATM-induced signaling in response to DNA damage.

EVIDENCE SUPPORTING PHOSPHORYLATION-INDUCED SUMO-DEPENDENT PROTEIN DEGRADATION

Promyelocytic leukemia is found to be degraded upon ATO-treatment and the degradation is dependent on the phosphorylation-induced by ATO and the subsequent increase of sumoylation (Lallemand-Breitenbach et al., 2001; Hayakawa and Privalsky, 2004). ATO-induced sumoylation on Lys-160 is critical for recruiting RNF4 to ubiquitylate PML for proteasomal degradation (Lallemand-Breitenbach et al., 2001; Petrie and Zelent, 2008). Although there is no direct link showing phosphorylation of PML promotes SUMO-dependent degradation, either phosphorylation-defective, or sumoylation-defective PML mutant shows abolished downstream effects in response to ATO-treatment (Lallemand-Breitenbach et al., 2001; Yang et al., 2002; Hayakawa and Privalsky, 2004), implying that both modifications and their crosstalk are indispensable in leading to ATO-induced PML degradation. PML can be phosphorylated by several kinases including MAPK, CK2, and CHK2 (Yang et al., 2002; Hayakawa and Privalsky, 2004; Joe et al., 2006; Scaglioni et al., 2006). Interestingly, phosphorylation-defective PML is stabilized upon DNA damage triggered by γ-irradiation and results in decreased apoptotic activity, in an ATM/CHK2-dependent manner (Yang et al., 2002). Evidence from the PML studies provide some hints supporting that the degradation promoted by RNF4 is possibly regulated by phosphorylation; however, it is still unclear how phosphorylation induces sumoylation of PML and whether there is another mechanism that phosphorylation of PML might enhance the recognition by RNF4.

UNIQUE STRUCTURAL CHARACTERISTIC OF RNF4 POSSIBLY LINKS PHOSPHORYLATION, SUMOYLATION AND UBIQUITYLATION

If one speculates that RNF4-mediated SUMO-targeted ubiquitylation is regulated by ATM-induced phosphorylation, what would the mode of regulation be? A unique region is found in RNF4 protein. Following the four SIMs, there is a region rich with arginine, named arginine-rich region (ARR) in RNF4 (**Figure 1**), denoting that this region provides positive charge to attract phosphorylated protein with negative charge. The electrostatic interaction between arginine and phosphate forms a covalent-like binding (Woods and Ferre, 2005). Thus, the ARR in RNF4 might enhance its interaction with target proteins phosphorylated by ATM. In summary, phosphorylation on RNF4 target proteins might be a mode to regulate RNF4-mediated, SUMO-targeted ubiquitylation and related biological function.

A POTENTIAL MECHANISM OF RNF4 TARGETING ATM SUBSTRATES FOR PROTEASOMAL DEGRADATION

It was noted that phosphorylation and subsequent sumoylation of PML occur within 1 h after treating with ATO (Lallemand-Breitenbach et al., 2001; Hayakawa and Privalsky, 2004); however, significant degradation of PML is observed around 12–16 h after ATO-treatment (Lallemand-Breitenbach et al., 2001, 2008), indicating that the regulation of RNF4-mediated PML degradation might be in slow kinetics. This suggests that there might be other factors required for targeting RNF4-ubiquitylated PML to

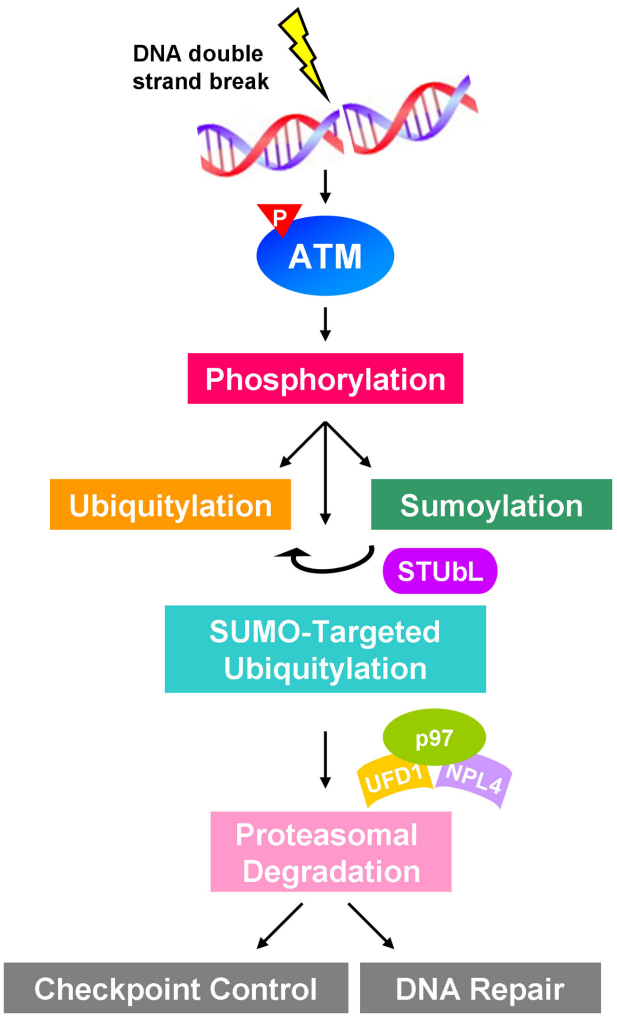

FIGURE 2 | Proposed schematic showing the crosstalk of phosphorylation, sumoylation, and ubiquitylation in ATM-regulated signaling in response to DNA damage. Double-strand breaks activate ATM to induce downstream signaling cascade involving sumoylation and ubiquitylation of proteins participating in DNA damage response, such as checkpoint control and DNA repair. STUbL functions as a signal transducer to coordinate SUMO and Ub signals to catalyze SUMO-targeted ubiquitylation, which may recruit p97–UFD1–NPL4 segregase complex to mediate the proteasomal degradation.

proteasome. An AAA-ATPase p97, its adaptors UFD1 and NPL4 are implicated in recognizing and extracting polyubiquitylated proteins to proteasome for degradation in various cellular context, including mitosis, DNA replication, and DNA damage (Richly et al., 2005; Ramadan et al., 2007; Mouysset et al., 2008; Meerang et al., 2011; Verma et al., 2011). To explain the observation of slow degradation of PML, rather than rapid turnover, in our view, RNF4 may serve as a signal transducer that senses SUMO signal and amplifies Ub signal on its substrates to recruit selective cargo proteins, such as p97–UFD1–NPL4 complex to extract the ubiquitylated substrates for proteasomal degradation.

CONCLUSION AND PERSPECTIVE

Sumoylation and ubiquitylation widely participate in ATM-regulated DDR. When cells are exposed to genotoxic stress, DSBs activate ATM to phosphorylate a subset of target proteins to transduce signals and to induce checkpoint control and DNA repair machinery. This process largely involves the cooperation of sumoylation and ubiquitylation to regulate cellular function in response to DSB. However, little is known about the detailed mechanism of SUMO–Ub crosstalk. Here, we hypothesize that RNF4 plays a central role in recognizing ATM-induced phosphorylation and sumoylation to provide an additional Ub signal to recruit Ub-selective segregase to target for proteasomal degradation (**Figure 2**). This provides a novel view on the crosstalk among multiple PTMs. The crosstalk of phosphorylation, sumoylation, and ubiquitylation denotes a cooperative network in protecting cells from DNA damage and maintaining genome integrity. Defects in this network may lead to genome instability and consequently tumorigenesis. Moreover, modulation of the players involved in the network may sensitize cancer cells to DNA damage-based cancer therapy and benefit the patients.

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CRL ubiquitin ligases and DNA damage response

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Cullin/RING ubiquitin ligases (CRL) comprise the largest subfamily of ubiquitin ligases. CRLs are involved in cell cycle regulation, DNA replication, DNA damage response (DDR), development, immune response, transcriptional regulation, circadian rhythm, viral infection, and protein quality control. One of the main functions of CRLs is to regulate the DDR, a fundamental signaling cascade that maintains genome integrity. In this review, we will discuss the regulation of CRL ubiquitin ligases and their roles in control of the DDR.

Keywords: DNA damage, cullin, Cdc25A, claspin, Cdt1, cyclin D1, ubiquitin ligase, protein degradation

INTRODUCTION

Our genome is under constant attack by exogenous DNA insulting agents, such as UV light and chemical carcinogens, and by endogenous metabolic products, such as reactive oxidative species. In order to prevent genome instability, a hallmark of cancers, eukaryotic cells have evolved several protective mechanisms of the DNA damage response (DDR), including DNA replication and cell cycle arrest, and DNA repair initiation (Harper and Elledge, 2007; Negrini et al., 2010). During a DDR, cascades of phosphorylation events are initiated by a family of PI3-like family kinases, mainly an ataxia telangiectasia mutated (ATM), and ATM and Rad3-related (ATR) kinases (Harper and Elledge, 2007; Negrini et al., 2010). Recent studies, however, have indicated that ubiquitination also plays important roles in the DDR (Harper and Elledge, 2007; Messick and Greenberg, 2009; Negrini et al., 2010; Silverman et al., 2012).

Ubiquitin is an 8-kDa protein and can be covalently conjugated to other proteins via an isopeptide linkage between its C-terminal glycine and a primary amino group of its substrates, which is usually from a lysine side chain (Pickart, 2004; Hannah and Zhou, 2009). Ubiquitination is catalyzed by three enzymes, including a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2), and a ubiquitin ligase (E3) (Pickart, 2004; Hannah and Zhou, 2009). The ubiquitination machinery can conjugate a single ubiquitin to one lysine residue of substrates (called mono-ubiquitination), multiple single ubiquitin molecules to multiple lysine residues (called multi-ubiquitination), or multiple ubiquitins in a chain (called poly-ubiquitination) to substrates. Mono-ubiquitination and multi-ubiquitination play important roles in endocytosis, DNA repair, immune response, and transcriptional regulation, etc. Poly-ubiquitination via surface lysine residues of ubiquitin, such as Lysine-48 (K48) or Lysine-11 (K11) often leads to protein proteolysis through the 26S proteasome (Pickart, 2004; Pickart and Eddins, 2004). However, protein poly-ubiquitination does not necessarily drive protein turnover. Poly-ubiquitin chains via Lysine-63 residue (K63) of ubiquitin and linear poly-ubiquitin chains appear to play non-proteolytic functions and are involved in DNA repair, NF-κB activation, and Ras localization and signaling, etc. (Pickart, 2004; Jura et al., 2006; Yan et al., 2009, 2010; Xu et al., 2010).

The human genome contains two E1 with distinctive functions (Chiu et al., 2007; Jin et al., 2007; Pelzer et al., 2007; Lee et al., 2011), dozens of E2s and hundreds of E3 ligases which determine the specificity of substrates for ubiquitination (Pickart, 2004; Pickart and Eddins, 2004). Thus far, more than 600 ubiquitin ligases have been identified in the human genome, although many of them have not been linked to any substrates or biological activities. Based on their domain characteristics, ubiquitin ligases can be separated into two sub-groups, HECT domain E3 ligases and RING finger domain E3 ligases (Pickart, 2004). The cullin/RING ubiquitin ligases (CRL) comprise the largest subfamily of RING finger-containing E3s (Petroski and Deshaies, 2005; Sarikas et al., 2011). CRLs are modular ubiquitin ligases (Petroski and Deshaies, 2005; Sarikas et al., 2011). CRLs are involved in cell cycle regulation, DNA replication, DDR, development, immune response, transcriptional regulation, circadian rhythm, viral infection, and protein quality control (Petroski and Deshaies, 2005). This review will focus on the interplay between human CRL ubiquitin ligases and the DDR. We will discuss molecular features of CRLs and how they participate in the DDR by targeting DDR-regulatory proteins for ubiquitination.

INTRODUCTION OF CRL UBIQUITIN LIGASES

Cullin/RING ubiquitin ligases consist of multiple subunits (Petroski and Deshaies, 2005; Sarikas et al., 2011), including a RING finger protein (Rbx1 or Rbx2) that recruits an activated ubiquitin

E2 enzyme, a scaffold subunit (cullin family proteins), and a substrate receptor that recognizes substrates. Many CRL ubiquitin ligases contain additional linker proteins, such as Skp1 in SCF (also called CRL1) and DDB1 in CRL4 (**Figure 1**). Recent studies indicate that Rbx1 associates with all cullins, except for Cul5, which associates specifically with Rbx2 (Kamura et al., 2004; Huang et al., 2009), suggesting distinct roles of the two Rbx homologs in human.

The first CRL E3 ligase, named SCF complex (stands for Skp1/Cdc53 or cullin/F-box), was identified from budding yeast, *Saccharomyces cerevisiae* (Bai et al., 1996; Willems et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997). This finding initiated with studies of proteasome-dependent turnover of Sic1, an inhibitor of cyclins and cyclin-dependent kinases (Cdks) in budding yeast (Bai et al., 1996; Willems et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997). Degradation and ubiquitination of Sic1 at the G1/S transition were compromised in Cdc4, Cdc53 (Cul1 homolog in yeast), and Skp1 mutants in budding yeast, which suggested their common roles in Sic1 turnover (Bai et al., 1996; Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997). The ubiquitination function of Cdc53 was observed in yeast Cln2 stability control as well (Willems et al., 1996). Biochemical evidence demonstrated that Cdc53 functions together with Cdc4 and Skp1 as a ubiquitin ligase (SCF^{Cdc4}) to catalyze the poly-ubiquitination of Sic1 both *in vitro* and *in vivo* (Feldman et al., 1997; Skowyra et al., 1997). Within the SCF complex, Cdc4, the F-box protein, is a substrate receptor that recognizes Sic1 within its WD-40 motif and interacts with Skp1 through its F-box domain. Skp1 is the linker protein that mediates association of Cdc4 with the scaffold cullin protein, Cdc53. Rbx1 (also called ROC1 or Hrt1 in yeast), a RING finger protein in the complex, was found to regulate the stability of yeast Sic1, human HIF1α, and other substrates (Lyapina et al., 1998; Kamura et al., 1999; Ohta et al., 1999; Seol et al., 1999; Skowyra et al., 1999; Tan et al., 1999). Structurally, Rbx1 binds to the C-terminal domain of yeast Cdc53 or human Cul1, and an E2 enzyme, Cdc34 (Zheng et al., 2002b; Zimmerman et al., 2010; Duda et al., 2011).

REGULATION OF CRL UBIQUITIN LIGASES

The activity of CRL ubiquitin ligases is regulated by NEDD8, a small ubiquitin-like protein (Deshaies et al., 2010). Like ubiquitin, NEDD8 can be conjugated to other proteins, especially cullins (Xirodimas et al., 2004; Watson et al., 2006; Jones et al., 2008).

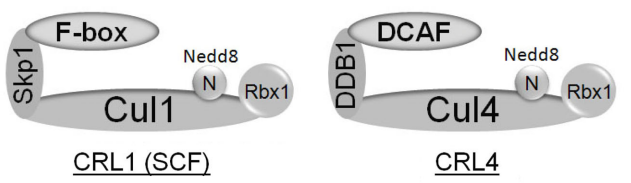

FIGURE 1 | Architecture of CRL1 (SCF) and CRL4 ubiquitin ligases. SCF ubiquitin ligases contain Cul1, Rbx1, Skp1, and F-box proteins which function as substrate receptors; whereas CRL4 has Cul4, Rbx1, DDB1, and DCAF proteins as substrate receptors. Both SCF and CRL4 can be activated by neddylation. F-box and DCAF proteins are substrate specificity factors.

The conjugation of NEDD8, named neddylation, is catalyzed by NEDD8-specific E1, E2, and E3 (Dye and Schulman, 2007). The general consensus is that neddylation of cullins is required for activation of CRL ubiquitin ligases. However, untimely neddylation of cullins could drive destruction of substrate receptors via auto-ubiquitination (Cope and Deshaies, 2006). Therefore, the neddylation of cullins is counteracted by the deneddylation activity of a multifunctional protein complex, the COP9 signalosome (CSN) (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wei et al., 2008).

COP9 signalosome was initially found to be involved in plant photo morphogenesis and was later identified as a conserved complex in eukaryotes (Cope and Deshaies, 2003; Serino and Deng, 2003; Wei and Deng, 2003; Wei et al., 2008). CSN is comprised of eight subunits, which are CSN1–8 in order of decreasing molecular weight. The CSN complex participates in multiple biological events, including transcriptional regulation, cell division, and development, etc. (Tateishi et al., 2001; Lykke-Andersen et al., 2003; Panattoni et al., 2008). Part of the multi-functionality of CSN is linked with the neddylation system, with its isopeptidase activity to remove NEDD8 conjugation. This deneddylation activity is attributed to the metalloprotease motif of CSN5 but the whole CSN complex is required for the reaction (Cope et al., 2002). Indeed, conditional silencing of CSN5 in HEK293 cells increased the neddylation of cullins. Consequently, expression of multiple F-box proteins, but not the cullins, was decreased (Cope and Deshaies, 2006). The reduced expression of F-box proteins depends on Cul1 and the proteasome, further supporting an auto-ubiquitination and self-destruction mechanism (Cope and Deshaies, 2006). These data explain why accumulation of CRL substrates has been observed in cells where either CSN or the NEDD8 conjugation system is inactivated (Tateishi et al., 2001; Lykke-Andersen et al., 2003; Cope and Deshaies, 2006; Panattoni et al., 2008; Choo et al., 2011). It is clear that expression of some substrate receptors is not affected by neddylation, however (Cope and Deshaies, 2006).

Neddylation of cullins positively regulates the E3 ligase activity of CRLs by at least three mechanisms. First, neddylation of cullins enhances their interaction with ubiquitin-activated E2. It has been suggested that neddylation of Cul1 can increase the affinity between ubiquitin-activated E2 and Rbx1 (Kawakami et al., 2001). Using fluorescence resonance energy transfer (FRET) technology, Saha and Deshaies (2008) observed that neddylation of Cul1 can enhance Cdc34 binding to SCF ubiquitin ligase.

Second, neddylation positively regulates the ubiquitination activity of CRL ubiquitin ligases by enhancing ubiquitin transfer to substrates from the active E2 site and by positioning the active E2 site closer to ubiquitin accepting sites of substrates. Neddylation has been shown to stimulate the Cdc34-dependent ubiquitination activity of SCF by more than 10-fold (Saha and Deshaies, 2008). The κ_{cat} for ubiquitin transfer is enhanced by NEDD8 conjugation (Saha and Deshaies, 2008). According to the structural analysis of the SCF^{Skp2} ubiquitin ligase, Cul1 holds the Skp1–Skp2 and Rbx1 subunits more than 100 Å apart (Zheng et al., 2002b). A gap of 50 Å between the active cysteine of E2 and the leucine-rich repeat of Skp2 exists even after E2 joins SCF^{Skp2} (Zheng et al., 2002b). This gap was confirmed by additional SCF structural studies (Wu et al.,

2003; Hao et al., 2007). In these scenarios, a gap of ∼50 Å is too large for E2 to transfer ubiquitin to its substrates. The neddylation of cullins, however, induces conformational changes in both the N-terminal and the C-terminal domains of cullins (Duda et al., 2008). These conformation changes make Rbx proteins acquire more flexibility to get access to their substrates. Such conformational rearrangements remain even after deneddylation, allowing both initiation and elongation of poly-ubiquitin chains.

Third, cullin neddylation prevents CRLs from associating with CAND1, a negative regulator of CRLs (Liu et al., 2002; Zheng et al., 2002a; Hwang et al., 2003; Min et al., 2003; Oshikawa et al., 2003; Goldenberg et al., 2004). The binding of CAND1 to cullins obscures their associations with their substrate receptors, which results in the inactivation of CRL E3 ligases. CAND1 only associates with non-neddylated cullins. Therefore, the popular model is that cullin neddylation favors the dissociation of CAND1 from CRLs. NEDD8-conjugated CRLs, which are free of CAND1, possess maximal E3 activity. After deneddylation by CSN, CAND1 is able to inhibit CRLs by binding to cullins. The next neddylation cycle can then prevent CAND1 from binding and CRL activity will resume. However, a recent study from the Harper group revealed that only a small fraction of cullins is associated with CAND1, whereas the majority of Cul1 proteins form complexes with F-box proteins, the substrate receptors (Bennett et al., 2010). Therefore, at least for the SCF ubiquitin ligases, the formation of SCF complexes is driven mainly by receptor binding.

Neddylation is catalyzed by its E3 ligase, Rbx (Huang et al., 2009). Recent studies indicated that the DCN family proteins function as additional E3 ligases to assist Rbx in cullin neddylation (Kurz et al., 2005, 2008; Ma et al., 2008; Meyer-Schaller et al., 2009). Wu et al. (2011) found that human DCN1 is modified by mono-ubiquitination, which drives nuclear export of DCN1. The biological impact of mono-ubiquitination of DCN1 under physiological condition remains to be determined, however. Interestingly, expression of DCN3L, a human homolog of yeast Dcn1p, can be enhanced by UV light (Ma et al., 2008), suggesting that the DNA damage signal may enhance neddylation of some, if not all, CRL ubiquitin ligases that are important for DDR.

The CRL family is one example of ubiquitin ligases that mediate many cellular signaling events (Petroski and Deshaies, 2005). In the following sections, we will discuss the roles of individual CRL ubiquitin ligases in control of DDR.

SCF$^{\beta TRCP}$ IN DNA DAMAGE CHECKPOINT CONTROL

SCF$^{\beta TRCP}$ IN DDR-INDUCED Cdc25A UBIQUITINATION AND TURNOVER

Cdc25A is a dual phosphatase that targets phosphorylated tyrosines and serines/threonines for de-phosphorylation (Karlsson-Rosenthal and Millar, 2006; Ray and Kiyokawa, 2007, 2008; Rudolph, 2007). Removal of inhibitory phosphate on tyrosine by Cdc25A activates cyclin/Cdk kinases, mainly cyclin E/Cdk2 and cyclin A/Cdk2, and enables the G1/S transition and mitotic entry. Cdc25A has two close homologs, Cdc25B and Cdc25C. It was proposed that Cdc25B initiates the activation of cyclin B/Cdk1 and Cdc25C completes this process in the nucleus during the G2/M transition (Boutros et al., 2006). However, both Cdc25B and Cdc25C are dispensable in mouse development, except for meiosis (Chen et al., 2001a; Ferguson et al., 2005). Cdc25B and Cdc25C double knockout mice are viable, but females are sterile, as are Cdc25B knockout females (Ferguson et al., 2005). In contrast, Cdc25A is essential for early embryo development (Ray et al., 2007a). In summary, Cdc25A is an essential gene for mouse development and is one of the major regulators of cell division (Molinari et al., 2000; Zhao et al., 2002; Ray and Kiyokawa, 2007, 2008). The Cdc25A protein level is tightly regulated via transcription and proteasome degradation in a cell cycle-dependent manner (Ray and Kiyokawa, 2007).

Over-expression of Cdc25A has been observed in multiple cancers (Kristjánsdóttir and Rudolph, 2004; Boutros et al., 2006). Studies in Cdc25A knockout mice indicated that Cdc25A is a rate-limiting oncogene that restricts tumorigenesis induced by the HER2/neu–RAS oncogenic pathway (Ray et al., 2007a). Consistent with this observation, MMTV-Cdc25A transgenic mice dramatically promote murine mammary tumorigenesis in cooperation with oncogenic RAS or neu (Ray et al., 2007b). Over-expression of Cdc25A in human cells can result in aberrant mitotic events, compromised DDR and destabilized chromosomes, such as chromosomal breaks at fragile sites (Cangi et al., 2008). Therefore, it is critical to maintain appropriate amounts of Cdc25A in human cells.

Cdc25A is an unstable protein and its protein levels are mediated by phosphorylation and the ubiquitin–proteasome pathway (Bernardi et al., 2000; Mailand et al., 2000, 2002; Falck et al., 2001; Donzelli et al., 2002, 2004; Shimuta et al., 2002; Zhao et al., 2002; Busino et al., 2003; Goloudina et al., 2003; Jin et al., 2003, 2008; Sørensen et al., 2003; Xiao et al., 2003; Kasahara et al., 2010). In response to DNA damage, Cdc25A is phosphorylated by the checkpoint kinase, Chk1, and subjected to proteasome-dependent degradation (Zhao et al., 2002; Goloudina et al., 2003; Jin et al., 2003; Sørensen et al., 2003; Xiao et al., 2003; Donzelli et al., 2004). The consequence of Cdc25A turnover is persistent Cdk phosphorylation and cell cycle arrest at S-phase upon DDR (Busino et al., 2003; Jin et al., 2003). IR-induced Cdc25A turnover in interphase was shown to depend on Cul1, suggesting the involvement of the SCF E3 ligase (Donzelli et al., 2002). In a screen to identify the substrate receptor of the SCF complex, the F-box proteins, βTRCP1 and βTRCP2, two homologs of human βTRCP, were found to be interacting with Cdc25A. Knockdown of both βTRCP1 and βTRCP2 by siRNA or expression of a dominant-negative βTRCP1 blocked DDR-induced Cdc25A proteolysis (Busino et al., 2003; Jin et al., 2003). In addition, depletion of both βTRCP1 and βTRCP2 results in hyperactive Cdk2 kinase activity and radioresistant DNA synthesis, which are characteristics of a defective intra-S-phase checkpoint (Busino et al., 2003; Jin et al., 2003).

The recognition of ubiquitin substrates by F-box proteins often requires post-translational modification, usually phosphorylation, on target proteins (Petroski and Deshaies, 2005). Chk1 kinase activity is indispensible for *in vitro* ubiquitination of Cdc25A catalyzed by SCF$^{\beta TRCP}$ (Jin et al., 2003). Upon DDR, the Serine-296 (S296) site of Chk1 is autophosphorylated after it is phosphorylated at Serine-345 (S345) by ATR (Kasahara et al., 2010). Chk1 phosphorylated at S296 is further recognized by 14-3-3 gamma. 14-3-3 gamma not only tethers Chk1 in the nucleus, but also bridges the interaction between Chk1 and Cdc25A (Kasahara et al., 2010). Therefore, 14-3-3 gamma is important for Chk1-mediated

Cdc25A turnover via the ubiquitin–proteasome pathway (Kasahara et al., 2010). Several Chk1 phosphorylation sites on Cdc25A were mapped, but only mutation on Serine-76 (S76) abolishes Cdc25A ubiquitination (Jin et al., 2003, 2008). Surprisingly, peptide scanning and mutagenesis revealed that S76 alone was not sufficient for direct binding with βTRCP (Jin et al., 2003). Adjacent to S76, there is a DSGFCLDSP sequence (residues 81–89) which resembles the classic phospho-degron motif in other βTRCP substrates (Cardozo and Pagano, 2004). Serine-88 is dispensable for $SCF^{\beta TRCP}$-mediated Cdc25A ubiquitination (Jin et al., 2003). Although Aspartic acid-87 could mimic a phosphorylated-serine, it appears not to be involved in binding of Cdc25A to βTRCP (Jin et al., 2003). Phosphorylation of both Serine-79 (S79) and Serine-82 (S82) is required for Cdc25A ubiquitination (Jin et al., 2003). In addition, the phospho-S79 and phospho-S82 peptides are able to bind to βTRCP *in vitro*, indicating these two sites are central to where βTRCP recognizes Cdc25A (Jin et al., 2003). Chk1 can phosphorylate neither S79 nor S82, however. Therefore, it seems that active Chk1 phosphorylates S76 of Cdc25A in response to DNA damage, which creates a priming site for additional kinases to subsequently phosphorylate S79 and S82 residues. Phosphorylation of these residues recruits $SCF^{\beta TRCP}$ to ubiquitinate Cdc25A for turnover (**Figure 2**).

Besides Chk1, another checkpoint kinase Chk2 plays an important role in DDR-induced Cdc25A degradation (Falck et al., 2001). Chk2 has been found to phosphorylate Serine-124 (S124) of human Cdc25A, which is important for IR-induced Cdc25A turnover. Compared to S76, however, S124 phosphorylation appears to play a minor role in DDR-induced degradation of Cdc25A (Jin et al., 2003). Moreover, Chk2 can not phosphorylate S76 of Cdc25A *in vitro* efficiently (Jin et al., 2008). These data may explain why deletion of Chk2 in HCT116 cells has no impact on IR-induced Cdc25A turnover (Jin et al., 2008). This further emphasizes the primary importance of Chk1 phosphorylation at S76 in control of Cdc25A under DDR. The discrepancy in the role of Chk2 in DNA damage-induced Cdc25A degradation may depend on cell types and different DNA damage signals. Further studies are needed to resolve this issue.

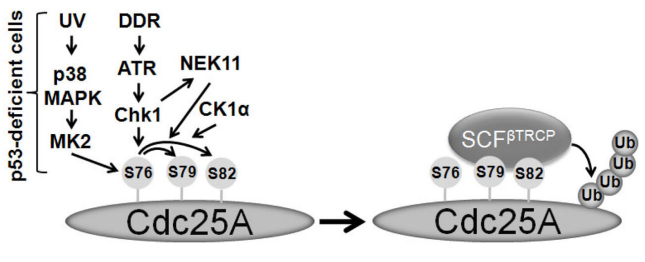

FIGURE 2 | Multiple kinases lead to the formation of phospho-degron of Cdc25A under DDR. Under DDR ATR activates Chk1 which then phosphorylates Cdc25A at S76 and turns on NEK11. Consequently, NEK11 phosphorylates Cdc25A at S82, whereas, CK1α phosphorylates both S79 and S82 of Cdc25A. In p53-deficient cells, p38MAPK and MK2 lead to the phosphorylation of S76 under UV damage. Upon the phospho-degron is created, Cdc25A is recognized and ubiquitinated by the $SCF^{\beta TRCP}$ ubiquitin ligase.

In addition to Chk1 and Chk2, other protein kinases are involved in Cdc25A proteolysis in unsynchronized or stressed cells as well (Reinhardt et al., 2007; Kang et al., 2008; Melixetian et al., 2009; Honaker and Piwnica-Worms, 2010; Myer et al., 2011; Piao et al., 2011). In p53-deficient cells, Cdc25A proteolysis depends on p38 MAP kinase (p38MAPK), and MAPKAP Kinase-2 (MK2), but not Chk1 (Reinhardt et al., 2007). Apparently, Chk1 and MK2 recognize similar phosphorylation motifs, such as the one surrounding S76 of Cdc25A (Reinhardt et al., 2007). In this case, p38MAPK and MK2 mediate a third DNA damage checkpoint pathway for cell cycle arrest and survival after DNA damage, although an intact ATR–Chk1 pathway exists in p53-deficient cells (Reinhardt et al., 2007). Therefore, p38MAPK and MK2 are potential candidates for drug targets to kill cancer cells that are defective in p53 function. However, how p53 affects the selection of checkpoint pathways in Cdc25A proteolysis has yet to be determined.

Chk1 and $SCF^{\beta TRCP}$ mediate Cdc25A turnover in the S and G2 phases of cell division. At late G1-phase, Cdc25A stability is not controlled by Chk1, although Cdc25A ubiquitination is still $SCF^{\beta TRCP}$-dependent. Kang et al. (2008) found that GSK3β phosphorylates S76 of Cdc25A at late G1-phase. Interestingly, S76 phosphorylation requires prior phosphorylation at Threonine-80 (T80) by PLK3 (Kang et al., 2008). A strong correlation between GSK3β inactivation and Cdc25A over-expression was observed in some human tumors, further supporting the negative role of GSK3β in Cdc25A production (Kang et al., 2008). However, under DDR, checkpoint kinases, such as Chk1 and MK2, are in charge of S76 phosphorylation of Cdc25A (Jin et al., 2003; Reinhardt et al., 2007).

S82 of Cdc25A is a primary phosphorylation site in the phospho-degron motif of Cdc25A. Several kinases have been reported to phosphorylate S82 to trigger Cdc25A turnover (Melixetian et al., 2009; Honaker and Piwnica-Worms, 2010; Piao et al., 2011). In a screen with a shRNA library, Melixetian et al. (2009) identified NIMA (never in mitosis gene A)-related kinase 11 (NEK11) as a gene that is involved in the G2/M checkpoint. Silencing NEK11 prevents ubiquitin-dependent proteolysis of Cdc25A, in both unsynchronized and DNA damaged cells. NEK11 phosphorylates S82 of Cdc25A during DDR. Moreover, NEK11 is a downstream effective kinase of the checkpoint pathway and is activated via phosphorylation at Serine-273 of NEK11 by Chk1. Thus far, there is no evidence to show whether NEK11 is capable of phosphorylating S79 of Cdc25A, although phosphorylation of S79 is important for S82 phosphorylation of Cdc25A and Cdc25A recognition by βTRCP. In a separate study, Honaker and Piwnica-Worms (2010) reported that casein kinase 1 alpha (CK1α) phosphorylates Cdc25A at both S79 and S82 during interphase and under genotoxic stress. In the same study, the authors claimed that NEK11 could not phosphorylate S82 of Cdc25A *in vitro*. However, it is unclear whether any priming kinase was included in the *in vitro* kinase assay. Paradoxically, Melixetian et al. (2009) found that NEK11 can phosphorylate S82 of Cdc25A *in vitro*, but the phosphorylation was independent of S76 phosphorylation. In contrast to the Honaker and Piwnica-Worms (2010) report, Piao et al. (2011) observed CK1ε directly phosphorylated S82 without any prior phosphorylation of Cdc25A, and silencing CK1ε stabilized

cellular Cdc25A in HEK293 cells. These conflicting results reveal the complexity of phosphorylation-regulated Cdc25A proteolysis (**Figure 2**). They may also reflect the fact that Cdc25A stability is regulated by various signaling pathways and that different genetic background may select distinctive kinases to create a phospho-degron motif for Cdc25A. Further experiments are needed to clarify these discrepancies. Nevertheless, the consensus is that S76 is a priming site for generation of a phospho-degron sequence surrounding S79 and S82.

In addition to residues surrounding S82, other serine residues also contribute to DDR-induced Cdc25A degradation. PLK3 phosphorylates Serines-513 and -519 of Cdc25A *in vitro* (Myer et al., 2011). The role of PLK3 in Cdc25A stability was studied in PLK3 knockout mice where the G1/S checkpoint was found to be defective (Myer et al., 2011). PLK3-mediated Cdc25A stabilization did not translate into a significant increase in tumorigenesis *in vivo* (Myer et al., 2011), however, suggesting that PLK3-mediated Cdc25A turnover may not be a major pathway to control *in vivo* functions of Cdc25A.

$SCF^{\beta TRCP}$ IN CLASPIN UBIQUITINATION AND TURNOVER DURING DDR RECOVERY

While $SCF^{\beta TRCP}$-mediated Cdc25A degradation is critical for the execution of the DNA damage checkpoint, $SCF^{\beta TRCP}$-controlled Claspin turnover is important for checkpoint recovery. Claspin associates with the replication fork and is one of the major checkpoint mediators for Chk1 activation by ATR in response to replication stress (Chini and Chen, 2003; Kumagai and Dunphy, 2003; Lee et al., 2003; Lin et al., 2004). Claspin is a periodically synthesized protein and its expression peaks at S/G2 and is then degraded after cells transit into mitosis. In unsynchronized cells, the mitotic degradation of Claspin depends on $SCF^{\beta TRCP}$, which binds to Claspin through a conserved DpSGxxpS phospho-degron motif (Cardozo and Pagano, 2004). Phosphorylation of these two serine residues is necessary for ubiquitination of Claspin. Alanine substitutions of these serine residues abolish the association of Claspin with $SCF^{\beta TRCP}$ and the poly-ubiquitination of Claspin (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Apparently, PLK1 is the kinase that phosphorylates these serines, because PLK1 is necessary for *in vitro* ubiquitination and siRNA depletion of PLK1 suppresses mitotic Claspin degradation (Mailand et al., 2006; Mamely et al., 2006).

In G2, Claspin quickly accumulates after UV damage, suggesting a post-transcriptional regulatory mechanism. The level of Claspin correlates well with activation of Chk1, represented by phosphorylation of Serine-317 of Chk1 (Chini et al., 2006). Chk1 phosphorylates Threonine-916 of Claspin and is important to maintain Claspin protein stability (Bennett and Clarke, 2006; Chini and Chen, 2006; Chini et al., 2006). Although Chk1 affects Claspin turnover, apparently, the stability control of Clapsin is independent of ATR (Chini and Chen, 2006; Chini et al., 2006; Yang et al., 2008). Conversely, Claspin is also a stabilizer of Chk1 protein (Yang et al., 2008). During the recovery from replication stress, Chk1 activity is gradually diminished, concomitantly with the reduction of Claspin level. When serine residues in the phospho-degron of Claspin are mutated into alanine, Claspin is no longer a substrate of the $SCF^{\beta TRCP}$ ubiquitin ligase (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Consequently, the activation of checkpoint pathway is extended and the percentage of mitotic cells is reduced (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006). Therefore, $SCF^{\beta TRCP}$-dependent turnover of Claspin is critical for cells to terminate the checkpoint pathway once the damage is repaired (Mailand et al., 2006; Mamely et al., 2006; Peschiaroli et al., 2006).

In summary, the $SCF^{\beta TRCP}$ ubiquitin ligase plays important roles in multiple steps of DDR via proteolytic control of multiple substrates which functions at different steps of DDR. Obviously, distinct protein kinases have been shown to create a conserved phospho-degron motif on different substrates. Nevertheless, as the substrate receptor, βTRCP is a key player to maintain genome integrity. Consistent with this, mutations of βTRCP1 have been identified in human cancer (He et al., 2005). The inhibitory role of βTRCP1 in tumorigenesis has been established (Kudo et al., 2004; Bhatia et al., 2011), suggesting that βTRCP1 is a *bona fide* tumor suppressor.

$CRL4^{Cdt2}$ IN DNA DAMAGE RESPONSE

DNA replication is initiated from the sequential recruitment and assembly of regulatory proteins onto specialized chromosome regions known as DNA replication origins. During the late M-phase and G1-phase, pre-replicative complex (pre-RC) needs to be assembled onto origins. Origins then become "licensed" and can further recruit DNA polymerase for replication at S-phase (Dutta and Bell, 1997; Arias and Walter, 2007). The assembly of pre-RC is a stepwise process that starts with binding of origin recognition complex (ORC) to an origin. ORC then recruits Cdc6 and Cdt1, two DNA replication licensing factors which are required for loading of MCM complex. To prevent re-replication, where origins fire more than once during a single cell cycle, it is essential to dissemble the pre-RC complex immediately after replication is initiated (Dutta and Bell, 1997; Arias and Walter, 2007). In *Xenopus* and mammals, Cdt1 can be inactivated through binding to its inhibitor, Geminin, or via ubiquitination-mediated proteolysis (Wohlschlegel et al., 2000; Nishitani et al., 2001; Tada et al., 2001; Arias and Walter, 2005). Inhibition of Geminin or over-expression of Cdt1 stimulates DNA re-replication (Vaziri et al., 2003; Zhu et al., 2004; Takeda et al., 2005). Cdt1 transgenic mice develop thymic lymphoblastic lymphoma in the absence of p53 (Seo et al., 2005) and over-expression of Cdt1 and Cdc6 promotes malignant events by inducing genome instability and by abrogating antitumor barriers, suggesting their oncogenic functions (Liontos et al., 2007). Over-expression of Cdt1 and/or Cdc6 has been observed in certain cancers (Karakaidos et al., 2004). Therefore, DNA replication licensing machine could be a good therapeutic target for cancer cures.

$CRL4^{Cdt2}$ IN DDR-INDUCED Cdt1 UBIQUITINATION AND TURNOVER

In the unperturbed cell cycle, Cdt1 is degraded after replication is initiated at G1-/S-phase (Nishitani et al., 2001). Walter and colleagues demonstrated that Cdt1 degradation in *Xenopus* egg extract requires replication initiation (Arias and Walter, 2005). Depletion of Cdc45 or RPA, which are essential for origin firing, abolished the chromatin-associated Cdt1 ubiquitination and degradation (Arias and Walter, 2005). Two E3 ligases, SCF^{Skp2} and $CRL4^{Cdt2}$, are identified to mediate cell cycle-dependent degradation of Cdt1 (Higa et al., 2003, 2006; Li et al., 2003; Zhong et al.,

2003; Hu et al., 2004; Kondo et al., 2004; Arias and Walter, 2006; Hu and Xiong, 2006; Jin et al., 2006; Kim and Kipreos, 2006; Lovejoy et al., 2006; Nishitani et al., 2006; Ralph et al., 2006; Sansam et al., 2006; Senga et al., 2006). SCF^{Skp2} is the main one to control Cdt1 throughout the cell cycle in a Cdk-dependent manner, whereas $CRL4^{Cdt2}$ is required for Cdt1 degradation in S-phase and during DDR.

In responding to UV or γ-irradiation, Cdt1 undergoes ubiquitination, and proteasome-dependent degradation, which presumably would prevent improper new origin firing before the damaged genome is repaired (Higa et al., 2003; Hu et al., 2004). The DDR-induced ubiquitination of Cdt1 is solely dependent on $CRL4^{Cdt2}$, since the absence of Skp2 does not compromise Cdt1 degradation after UV. Deletion of Cdt2 in HeLa cells induced DNA re-replication and G2/M checkpoint activation, partly due to Cdt1 accumulation (Jin et al., 2006; Sansam et al., 2006). How $CRL4^{Cdt2}$ recognizes and targets Cdt1 for ubiquitination has been intensively investigated. The degron signal recognized by $CRL4^{Cdt2}$ is localized at the N-terminus of Cdt1 since the N-terminal fragment behaves like the full length Cdt1 in the aspect of UV-induced degradation. Truncation mutagenesis further narrows the degron region to be within the first 10 residues, which also encompass a PCNA-interacting motif (called PIP box). There are evidences indicating the essential role of PCNA in Cdt1 degradation (Arias and Walter, 2006; Hu and Xiong, 2006; Senga et al., 2006). Depletion of PCNA or mutation on the PIP box abolishes $CRL4^{Cdt2}$-mediating Cdt1 ubiquitination and proteolysis in the unperturbed S-phase or UV damaged cells (Arias and Walter, 2006; Hu and Xiong, 2006; Senga et al., 2006). The Walter group further found that a TD motif inside the PIP box and a basic amino acid at four residues downstream of the PIP box of Cdt1 are important for Cdt1 to interact with PCNA strongly and to recruit $CRL4^{Cdt2}$ to the Cdt1–PCNA complex (Havens and Walter, 2009). This observation was confirmed by two individual studies (Guarino et al., 2011; Michishita et al., 2011).

Although both UV and IR can trigger $CRL4^{Cdt2}$-dependent Cdt1 degradation, the phosphorylation event of Cdt1 is only observed in UV damaged cells (Kondo et al., 2004). IR-induced degradation of Cdt1 is not dependent on either ATM or ATR checkpoint pathway, whereas UV-induced degradation is sensitive to the inhibitor of ATM and ATR, caffeine (Higa et al., 2003; Kondo et al., 2004). Although Cdt1 is phosphorylated after UV irradiation, none of the phosphorylation events are located at the N-terminus, where the degron motif is localized (Kondo et al., 2004; Senga et al., 2006). However, it is still unclear why caffeine, as the inhibitor of ATM and ATR, only affects UV-mediated Cdt1 proteolysis. How ATM and ATR regulate UV-controlled Cdt1 degradation is still unclear. Further lost-of-function investigations on ATM and ATR are necessary to validate their roles in UV-triggered Cdt1 proteolysis. Moreover, whether and how the phosphorylations of Cdt1 at its C-terminus are linked to Cdt1 ubiquitination and turnover are yet to be determined. Recent study from Cook's group indicated that p38MAPK and c-Jun N-terminal kinase (JNK) can phosphorylate Cdt1 both during unperturbed G2 phase and under stress condition (Chandrasekaran et al., 2011). These results explained why Cdt1 becomes a stable protein when cells move into G2 and mitosis.

$CRL4^{Cdt2}$ IN DDR-INDUCED p21 UBIQUITINATION AND TURNOVER

$CRL4^{Cdt2}$ is a key ubiquitin ligase that mediates ubiquitination of several important cell cycle regulators in cooperation with PCNA. The Cdk inhibitor, p21 (also called WAF1 or CIP1), is a substrate of $CRL4^{Cdt2}$ in DNA replication licensing control. p21 is an inhibitor of Cdks whose kinase activity is required to drive Cdc6 out of the nucleus in order to prevent DNA replication relicensing (Kim et al., 2007). p21 binds to the cyclin/Cdk2 complex to suppress its kinase activity and prevents cells from entering into S-phase. In response to different stimuli, the transcription of p21 is activated by p53, through which cell cycle progression is arrested at the G1/S transition. In addition to functioning as a cell cycle regulator, p21 also forms a complex with PCNA and polymerase δ. Over-expression of p21 disrupts the PCNA–Fen1 interaction *in vivo* and potentially interferes with the DNA repair process (Chen et al., 1996).

Under low dose UV irradiation, p21 undergoes proteolysis, which could potentially promote DNA repair. Two E3 ligases, SCF^{Skp2} and $CRL4^{Cdt2}$ are implicated in regulating p21 proteolysis under DNA damage conditions (Bornstein et al., 2003; Soria et al., 2006; Abbas et al., 2008; Kim et al., 2008; Nishitani et al., 2008). It has been shown that degradation of p21 after low dose UV irradiation ($<40\ J/m^2$) depends on ATR and Skp2. The interaction between Skp2 and p21 has been detected and is further increased after DNA damage (Bendjennat et al., 2003). Mutation of all lysine residues impaired UV-induced p21 degradation and cells exhibited defects in DNA repair. This implies that p21 ubiquitination promotes its degradation to prompt the accumulation of PCNA on chromatin for DNA repair process (Bendjennat et al., 2003).

p21, like Cdt1, has a PIP box motif for PCNA interaction. It was then speculated whether p21 degradation could be regulated like Cdt1, via $CRL4^{Cdt2}$-mediated ubiquitination with PCNA as co-factor. Down-regulation of Cul4, DDB2, and Cdt2 in UV-irradiated p53 null HCT116 (to eliminate the signaling from p53) inhibited efficient p21 degradation (Abbas et al., 2008). As in the case for Cdt1, p21 ubiquitination by $CRL4^{Cdt2}$ requires PCNA binding via the PIP box. The association of p21 and $CRL4^{Cdt2}$ was detected by co-immunoprecipitation regardless of damage signaling. However, $CRL4^{Cdt2}$ can only ubiquitinate p21 that possesses a phosphomimetic mutation at Serine-114, which is the target of the GSK3β kinase stimulated by ATR (Abbas et al., 2008).

The classic E2 enzyme for CRL ubiquitin ligases is Cdc34 (Petroski and Deshaies, 2005). A recent study, however, indicated that UbcH5 may function as a priming E2 to initiate ubiquitination of IκBα, a *bona fide* substrate of the $SCF^{\beta TRCP}$ ubiquitin ligase (Wu et al., 2010). Given the fact that CRLs share a common RING finger protein, Rbx, it is highly possible that the E2 priming step by UbcH5 may be common to poly-ubiquitination of many, if not all substrates of CRLs. Interestingly, the Dutta group found that UbcH8 mediates p21 ubiquitination, whereas the UBE2G family of E2s cooperate with $CRL4^{Cdt2}$ to polyubiquitylate Cdt1 under DDR (Shibata et al., 2011). These data, if true, suggested that substrates also play important roles in E2 selection of CRL ubiquitin ligases. Because these experiments were done with single siRNA oligo, further studies are needed to confirm the roles of these E2 enzymes. UbcH8 lacks obvious features for poly-ubiquitin chain formation. Whether other E2s, such as UbcH5, can function together with

UbcH8 in poly-ubiquitin chain generation on p21 remains to be determined. Moreover, like Cdc34, UBE2G E2s contain an acidic sequence that is required for poly-ubiquitin chain formation (Petroski and Deshaies, 2005). One important question is whether UBE2G, like Cdc34, needs assistance from another E2, such as UbcH5, as a priming subunit to boost its poly-ubiquitination capability. Another critical question is whether the $CRL4^{Cdt2}$ ubiquitin ligase employs Cdc34, UbcH8, or UBE2G as E2 enzyme to conjugate poly-ubiquitin chains on Set8/PR-Set7, another critical substrate of $CRL4^{Cdt2}$ under DDR (Abbas et al., 2010; Centore et al., 2010; Oda et al., 2010; Jørgensen et al., 2011). Understanding these questions is important to better comprehend protein poly-ubiquitination by CRL ubiquitin ligases under DDR.

$CRL4^{DDB2}$ AND $CRL4^{CSA}$ IN DNA REPAIR CONTROL

Nucleotide excision repair (NER) is a versatile repair mechanism to remove the damaged DNA lesions from the genome, including UV-induced cyclobutane pyrimidine dimers (CPD), 6–4 pyrimidine–pyrimidone photoproducts, and a variety of bulky adducts (Gillet and Schärer, 2006). It is a gap-filling process executed by DNA polymerase and ligase following the excision of a 24–32 DNA base oligonucleotide, where the lesion localizes. There are two sub-types of NER, global genome repair (GGR), and transcription-coupled repair (TCR), which differ in the mechanism of damage detection. GGR occurs throughout the whole genome, whereas TCR is restricted in the active transcribing regions. Mutations in the NER pathway are responsible for several rare inheritable diseases, such as xeroderma pigmentosum (XP) and Cockayne syndrome (Bootsma et al., 1998).

$CRL4^{DDB2}$ IN NUCLEOTIDE EXCISION REPAIR

Xeroderma pigmentosum patients are hypersensitive to UV and are pre-disposed to skin cancer. Cells from XP patients are defective in repair of UV-induced DNA damage (Friedberg, 1995; Friedberg et al., 1995). In XP complementation group E (XP-E), the DDB2 gene is mutated. DDB2 encodes a WD-40 repeat protein that was originally identified as one subunit of a heterodimer with DDB1 (Dualan et al., 1995). The DDB1–DDB2 heterodimer binds to damaged DNA and is involved in NER. The crystal structure of DDB2–DDB1 dimer explains its high affinity toward UV-induced DNA lesion and its central role in NER. The hairpin motif of DDB2 inserts into the minor groove of a DNA duplex and thereby flips out the pyrimidine dimer to be recognized by the binding pocket of DDB2 (Scrima et al., 2008). The DNA lesion recognition ability of DDB2 is essential for subsequent recruitment of repair proteins to the damage site. Consistent with the clinical implication of DDB2 in human disease (XP-E), DDB2 knockout mice are susceptible to UV-induced skin tumorigenesis and develop spontaneous cancers at old age (Yoon et al., 2005).

DDB1 and DDB2 are two essential subunits of the $CRL4^{DDB2}$ ubiquitin ligase where DDB2 functions as the substrate receptor (Groisman et al., 2003). Thus far, the known substrates for $CRL4^{DDB2}$ are histones and XPC (Sugasawa et al., 2005; El-Mahdy et al., 2006; Kapetanaki et al., 2006; Wang et al., 2006; Guerrero-Santoro et al., 2008). $CRL4^{DDB2}$ is not so active due to its association with the CSN (Groisman et al., 2003). However, UV radiation can trigger the translocation of $CRL4^{DDB2}$ to chromatin. The recruitment of $CRL4^{DDB2}$ is critical for orchestrating the repair machinery around the damage site. Chromatin-bound $CRL4^{DDB2}$ is very active, because it does not associate with the CSN and its Cul4 subunit is neddylated (Groisman et al., 2003; Takedachi et al., 2010). Apparently, UV light sends signals to separate the CSN from $CRL4^{DDB2}$, because the majority of $CRL4^{DDB2}$ binds to chromatin after UV radiation (Groisman et al., 2003). Some DDB2 mutants from XP-E patients are defective in DDB1 binding (Jin et al., 2006; Takedachi et al., 2010). These mutants fail to recruit the other subunits of $CRL4^{DDB2}$ to lesion DNA under DDR (Hwang et al., 1998; Shiyanov et al., 1999; Jin et al., 2006; Kapetanaki et al., 2006; Takedachi et al., 2010). As a result, XPC and histones are not ubiquitinated (Kapetanaki et al., 2006; Takedachi et al., 2010). This may explain how the DNA repair machine is impaired in some XP-E patients.

XPC is a central player in the NER pathway. XPC is necessary for the assembly of NER complex surrounding the lesion (Sugasawa, 2011), whereas its DNA binding affinity is less than that of DDB2. It has been shown that the poly-ubiquitination of XPC after UV treatment depends on the $CRL4^{DDB2}$ ubiquitin ligase and that ubiquitination can augment the DNA binding affinity of XPC instead of targeting it to the proteasome for degradation (Batty et al., 2000; Sugasawa et al., 2005). One important question is why poly-ubiquitination of XPC by $CRL4^{DDB2}$ does not lead to XPC proteolysis?

The poly-ubiquitination of DDB2 reduces its DNA binding ability and leads to its degradation (Chen et al., 2001b; Nag et al., 2001; Matsuda et al., 2005). Silencing of Cul4A was shown to stabilize DDB2, to prolong the retention of DDB2 at UV-induced lesions, and to impair the recruitment of XPC and the subsequent removal of CPD from the genome (El-Mahdy et al., 2006). Moreover, XPC ubiquitination may accelerate DDB2 turnover (Sugasawa et al., 2009). Therefore, it seems likely that a UV-induced DNA lesion is handed over from DDB2 to XPC for DNA repair and that this process depends on the poly-ubiquitination capability of the $CRL4^{DDB2}$ ubiquitin ligase (Sugasawa et al., 2009). Whether XPC ubiquitination triggers DDB2 degradation via auto-ubiquitination is still debatable. However, a recent study from *Arabidopsis* suggested that DET1 is the substrate receptor of CRL4 that targets DDB2 for ubiquitination and degradation (Castells et al., 2011). Therefore, it will be interesting to see whether DET1 is responsible for UV- and Cul4A-mediated DDB2 ubiquitination and proteolysis in human cells. Using a chemical inhibitor, Zhao et al. (2008) demonstrated that p38MAPK is required for UV-induced DDB2 ubiquitination and proteolysis. The same group also found that DDB2 is phosphorylated by p38MAPK after UV treatment. However, the actual phosphorylation sites of DDB2 are yet to be identified. Moreover, it is still unclear whether phosphorylation of DDB2 under UV damage plays any critical roles in DDB2 ubiquitination and degradation. It is also unknown whether p38MAPK phosphorylates DDB2 directly or via its downstream kinases, such as MK2. Answering these questions will help understand the concise mechanism of NER under DDR.

In addition to XPC, histone proteins, H2A, H3, and H4, are conjugated with a single ubiquitin by $CRL4^{DDB2}$ in response to UV irradiation. In XP-E patient-derived lymphoblastoid cells, UV-induced mono-ubiquitination of H2A is impaired (Kapetanaki

et al., 2006). On one hand, some DDB2 mutants lose their functions as ubiquitin ligases, because they fail to interact with DDB1 (Jin et al., 2006; Kapetanaki et al., 2006). On the other hand, the K244E mutant of DDB2 contains only residual binding ability to DNA lesions (Takedachi et al., 2010). Although K244E maintains the ubiquitination activity of $CRL4^{DDB2}$, it fails to ubiquitinate histones in nucleosome context (Takedachi et al., 2010). It has been proposed that ubiquitination of histone proteins by $CRL4^{DDB2}$ loosens up the condensed chromatin structure into a relaxation state that is more accessible to the repair factors (Wang et al., 2006). However, inconsistent with this hypothesis, histone ubiquitination is not sufficient to destabilize the nucleosome *in vitro* (Takedachi et al., 2010). Therefore, one potential function of histone ubiquitination is to recruit other DNA repair factors to lesion DNA. One such factor could be XPA whose association with lesion DNA is enhanced by the ubiquitination activity of $CRL4^{DDB2}$ (Takedachi et al., 2010). However, XPA does have an obvious ubiquitin-binding motif, suggesting that additional factors might be involved in the recruitment of XPA. CRLs usually conjugate poly-ubiquitin chains on their substrates (Petroski and Deshaies, 2005). How $CRL4^{DDB2}$ is regulated to conjugate mono- versus poly-ubiquitin chains on its substrates remains enigmatic.

$CRL4^{CSA}$ IN NUCLEOTIDE EXCISION REPAIR

Besides $CRL4^{DDB2}$, the $CRL4^{CSA}$ ubiquitin ligase is also actively involved in DNA repair (Groisman et al., 2003). In this complex, CSA is a substrate specific factor. CSA is also a WD-40 repeat protein and has 40% sequence homology with DDB2 from the second to the fifth WD-40 repeat. Similar to DDB2, CSA directly binds to DDB1 and is able to assemble an E3 ligase complex with Cul4 and Rbx1. In response to UV damage, CSA is found associating with the stalled RNA polymerase II (RNA pol II) (Groisman et al., 2003). Similar to $CRL4^{DDB2}$, the ubiquitin ligase activity of $CRL4^{CSA}$ is inhibited due to the binding of CSN (Groisman et al., 2003). CSB, a DNA-dependent ATPase, is the only substrate identified for $CRL4^{CSA}$ thus far. CSB preferentially associates with elongating or stalled RNA pol II, and is potentially involved in the elongation step of RNA synthesis. Both CSA and CSB are mutated in Cockayne syndrome patients and involved in transcription-coupled DNA repair (Li et al., 2011). CSB fibroblasts exhibit hypersensitivity to UV irradiation and a defect in resumption of RNA synthesis after damage (Bregman et al., 1996; Balajee et al., 1997). Moreover, poly-ubiquitination and degradation of stalled RNA pol II in response to UV is also impaired in CSB cells (Bregman et al., 1996). CSB is a member of the SNF2-like family, which possesses chromatin-remodeling activity. CSB is believed to push away the stalled RNA pol II or to rearrange the chromatin structure so that the repair proteins can get access to the lesion (Groisman et al., 2006). The key function of CSB is to recruit histone acetyltransferase p300, NER proteins, and the $CRL4^{CSA}$ ubiquitin ligase to lesion DNA (van Gool et al., 1997; Fousteri et al., 2006); whereas, CSA is required to recruit XAB2, the nucleosomal binding protein HMGN1, and TFIIS in cooperation with CSB. If the lesion cannot be repaired, CSB then initiates poly-ubiquitination and degradation of stalled RNA pol II (Svejstrup, 2003). Three hours after damage, CSB itself is eliminated via $CRL4^{CSA}$-mediated poly-ubiquitination and proteasome-dependent degradation (Groisman et al., 2006). The degradation of CSB is necessary for transcription to resume at a normal rate, and is coordinated with the dissociation of the CSN from the $CRL4^{CSA}$ ubiquitin ligase (Groisman et al., 2003), indicating that the ubiquitination function of $CRL4^{CSA}$ was inhibited during DDR, but activated after DNA repair was accomplished. However, how the CSN is released from the $CRL4^{CSA}$ ubiquitin ligase is still unclear.

In summary, $CRL4^{CSA}$ and $CRL4^{DDB2}$ are two important ubiquitin ligases that not only function as ubiquitin ligases to ubiquitinate their cognate substrates, but also play distinct roles in recruiting critical DNA repair factors to lesion DNA upon DDR. Multiple mutations have been identified for DDB2 and CSA from human patients. Interestingly, most of these mutants fail to form ubiquitin ligases with other subunits of CRL4 (Jin et al., 2006). As a consequence, CRL4 ubiquitin ligases cannot be recruited to damage sites to assist DNA repair. Therefore, these disease-derived mutations all affect activities of $CRL4^{CSA}$ and $CRL4^{DDB2}$ ubiquitin ligases, further supporting their fundamental roles in NER.

SCF^{Fbxo4} AND SCF^{Fbxo31} IN CYCLIN D1 UBIQUITINATION

Cdk kinase activity is the master regulator that controls cell cycle progression. Cdk kinase activity can be modulated via phosphorylation, through binding to the inhibitors, or by association with cyclin co-factors. The level of cyclin proteins fluctuates during the cell cycle through periodic transcriptional activation or proteolysis. Cyclin D1 is expressed in G1-phase and its presence activates Cdk2 for entrance into S-phase. To prevent cells from entering into S-phase and propagating erroneous DNA, cyclin D1 is degraded via the ubiquitin–proteasome pathway after encountering genotoxic stress (Pagano et al., 1994; Diehl et al., 1997, 1998). Cyclin D1 is over-expressed in various malignant neoplasms, suggesting its role as an oncogene (Malumbres and Barbacid, 2001). MMTV-cyclin D1 transgenic mice develop mammary adenocarcinomas, indicating its oncogenic role in breast cancer (Wang et al., 1994; Hosokawa et al., 2001). Therefore, cyclin D1 expression is well controlled during cell division. Dysregulation of cyclin D1 can lead to genomic instability (Pontano et al., 2008). Cyclin D1 is an unstable protein. Its ubiquitination and degradation depends on phosphorylation of Threonine-286 (T286), which is mediated by GSK3β kinase (Diehl et al., 1997, 1998). Four F-box proteins, including Fbxo4, Fbxw8, Fbxo31, and Skp2, have been reported to recognize cyclin D1 and mediate its ubiquitination (Yu et al., 1998; Lin et al., 2006; Okabe et al., 2006; Barbash et al., 2009; Santra et al., 2009).

When cells progress into S-phase, the T286 on cyclin D1 is phosphorylated by GSK3β. Phosphorylated T286 and surrounding residues constitute the phospho-degron motif that is recognized by Fbxo4, a specificity factor in the SCF^{Fbxo4} ubiquitin ligase (Diehl et al., 1997, 1998; Lin et al., 2006). Thus, phosphorylated cyclin D1 at T286 is an unstable protein that is subjected to poly-ubiquitination by SCF^{Fbxo4} and then degradation by the 26S proteosome (Lin et al., 2006). Importantly, the ubiquitination of cyclin D1 by SCF^{Fbxo4} requires a small heat-shock protein, αB crystallin (Lin et al., 2006). Over-expression of Fbxo4 and αB crystallin can stimulate cyclin D1 ubiquitination and accelerate its degradation, whereas knockdown of either of them can block cyclin D1 ubiquitination and increase its protein stability (Lin

et al., 2006). In consistent with these observations, Fbxo4 and αB crystallin expression is reduced in subset of primary human tumors that overexpress cyclin D1 (Lin et al., 2006). In addition to Fbxo4, another F-box protein Fbxw8 was also identified as an E3 ligase for cyclin D1 in HCT116 cancer cell line. Fbxw8 can associate with either Cul1 or Cul7 to assemble an SCF or SCF-like complex, respectively (Okabe et al., 2006). Fbxw8 also recognizes the degron sequences surrounding phosphorylated T286. However, the phosphorylation of T286 in this case is mediated via the MAPK pathway rather than by GSK3β in the cell context examined (Okabe et al., 2006). The access of cyclin D1 to its cognate E3 ligase is spatially regulated. Similar to Fbxo4, majority of Fbxw8 is localized in the cytoplasm during G1- and S-phase, separating away from the nucleus localized cyclin D1. In S-phase, cyclin D1 is translocated into the cytoplasm, where it is now accessible to E3 ligase for subsequent ubiquitination and proteolysis. The phosphorylation of cyclin D1 by GSK3β at T286 is required for its nuclear export (Lin et al., 2006).

In contrast to Fbxo4 and Fbxw8, Fbxo31 is specifically dedicated to damage-induced degradation of cyclin D1 (Santra et al., 2009). In response to IR or other genotoxic stress, Fbxo31 is targeted by the checkpoint kinase ATM for phosphorylation, which results in the accumulation of Fbxo31. Fbxo31 binds to the same phospho-degron motif on cyclin D1 as Fbxo4 and Fbxw8 and mutation of T286 abolishes the interaction between cyclin D1 and all three F-box proteins. However, blocking of the MAPK pathway but not GSK3β prevents this damage-induced cyclin D1 degradation (Santra et al., 2009). It should be noted that the characterization of these four F-box proteins were done in different cell lines. Whether these four E3 ligases are involved in regulating cyclin D1 simultaneously or exclusively from each other remain to be determined. The Fbxo4 knockout mice develop various tumors whose cyclin D1 expression is enhanced (Vaites et al., 2011). However, a recent study in knockout mice of these ubiquitin ligases indicated that none of these four ubiquitin ligases is required for cyclin D1 proteolysis in mouse embryonic fibroblasts (Kanie et al., 2012), further supporting cell type-specific regulation of cyclin D1 stability. Therefore, it is intriguing that how cells choose among different ubiquitin ligases reacting to different stimuli for cyclin D1 destruction if they all recognize the same phospho-degron motif. Nevertheless, loss of heterozygosity of Fbxo31 has been reported in multiple cancers (Launonen et al., 2000; Lin et al., 2001; Härkönen et al., 2005; Kumar et al., 2005; Huang et al., 2010; Kogo et al., 2011). Inactive mutations of Fbxo4 are also identified in 15% of esophageal tumors (Barbash et al., 2008). These data support the idea that Fbxo31 and Fbxo4 are tumor suppressors.

CONCLUSION AND PROSPECTIVE

Here we discuss how CRL ubiquitin ligases mediate protein ubiquitination and turnover to enforce unidirectional signal transduction in the DDR pathway. Many of the substrate receptors of CRL are still uncharacterized. Growing evidence suggests that more CRL ubiquitin ligases will be found to mediate the DDR pathway and more DDR-related proteins will be identified as ubiquitin substrates of CRLs. Thus far, the main research focus has been on dissecting the roles of CRL1 (SCF) and CRL4 ubiquitin ligases in DDR. Emerging evidence suggests that other cullin-related ubiquitin ligases are also involved in DDR (Ribar et al., 2007; Yasukawa et al., 2008; Blackford et al., 2010). Further investigation is needed to explore the functions of cullin-related ubiquitin ligases other than CRL1 and CRL4 in DDR. Moreover, several groups have reported knockout mice models of Cul4A and suggest an important role of Cul4A in guarding the genome stability (Kopanja et al., 2009; Liu et al., 2009). The homolog of Cul4A, Cul4B shares significant sequence homology with Cul4A and has some redundant functions with Cul4A (Higa et al., 2003; Hu et al., 2004). Considering the distinct knockout phenotype of Cul4A in mice and cell lines (Nishitani et al., 2001; Karakaidos et al., 2004), it is conceivable that Cul4A and Cul4B play distinct roles in genome integrity maintenance via regulating protein stability of different substrates. However, Cul4B also plays significant roles in DDR (Kerzendorfer et al., 2010). In fact, degradation of p27 and p53 seems to depend on Cul4A solely (Banks et al., 2006; Higa et al., 2006). Therefore, more investigation is required to unravel the relationship between Cul4A and Cul4B and to advance our understanding of Cul4 as genome guardian.

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Histone ubiquitination and deubiquitination in transcription, DNA damage response, and cancer

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Histone post-transcriptional modifications play essential roles in regulation of all DNA related processes. Among them, histone ubiquitination has been discovered for more than three decades. However, its functions are still less well understood than other histone modifications such as methylation and acetylation. In this review, we will summarize our current understanding of histone ubiquitination and deubiquitination. In particular, we will focus on how they are regulated by histone ubiquitin ligases and deubiquitinating enzymes. We will then discuss the roles of histone ubiquitination in transcription and DNA damage response and the crosstalk between histone ubiquitination and other histone modifications. Finally, we will review the important roles of histone ubiquitination in stem cell biology and cancer.

Keywords: ubiquitin ligases, deubiquitinating enzymes, DUBs, H2Aub, H2Bub, BRCA1, RNF20, USP22

INTRODUCTION

In the eukaryotic nucleus, genomic DNA is packaged into chromatin by forming nucleosomes. Each nucleosome core particle consists of a histone octamer which is wrapped by 146 base pairs of DNA (Luger et al., 1997). A histone octamer is composed of two copies of the core histones H2A, H2B, H3, and H4. The histone tails protrude from the nucleosome, and are subjected to a wide array of covalent modifications include methylation, acetylation, ubiquitination, phosphorylation, sumoylation, and ADP ribosylation (Strahl and Allis, 2000). These post-transcriptional modifications work together to regulate the chromatin structure, which affects biological processes including gene expression, DNA repair, and chromosome condensation. Recent advances have defined critical roles of histone ubiquitination in transcriptional regulation and DNA repair. The writers, erasers, and readers of histone ubiquitination have also been linked to cancer development.

HISTONE UBIQUITINATION

Histone H2A is the first protein identified to be modified by ubiquitin in cells (Goldknopf et al., 1975). We know now H2A and H2B are two of the most abundant ubiquitinated proteins in the nucleus. It is estimated that 5–15% of H2A and 1–2% of H2B are conjugated with ubiquitin in vertebrate cells, while about 10% of H2B are ubiquitinated in yeast cells (Goldknopf et al., 1975; Matsui et al., 1979; West and Bonner, 1980; Robzyk et al., 2000).

The dominant form of ubiquitinated histones are monoubiquitinated H2A (H2Aub) and H2B (H2Bub). A single molecule of ubiquitin is added to the highly conserved lysine residues: Lys-119 for H2A, and Lys-123 in yeast or Lys-120 in vertebrate for H2B (**Figure 1**; Goldknopf et al., 1975; West and Bonner, 1980). Chromatin immunoprecipitation (ChIP) experiments showed that monoubiquitinated H2A is enriched in the satellite regions of genome, while H2Bub binds to the gene body of transcriptional active genes (Minsky et al., 2008; Shema et al., 2008; Zhu et al., 2011).

In addition to H2A and H2B, core histones H3, H4, and linker histone H1 have also been reported to be modified by ubiquitin (Pham and Sauer, 2000; Jason et al., 2002; Wang et al., 2006; Jones et al., 2011). For example, H3 and H4 were polyubiquitinated *in vivo* by CUL4–DDB–RBX1 ubiquitin ligase complex after UV irradiation (Wang et al., 2006). But the biological function of these modifications has not been well elucidated.

Besides monoubiquitination, histone H2A and H2B can be modified by ubiquitin chains. K63-linked polyubiquitination of H2A and H2AX, a variant of H2A, is usually induced by DNA damage and is required for DNA repair response (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007; Doil et al., 2009; Stewart et al., 2009). Like other proteins, formation of K48-linked ubiquitin chains on histones targets them for proteasome mediated degradation. For example, during spermatogenesis, histones are degraded through this mechanism and replaced by transition proteins to permit chromatin condensation (Chen et al., 1998; Liu et al., 2005).

HISTONE UBIQUITINATION ENZYMES

In this section, we will summarize our current knowledge on the histone modifying enzymes that can add ubiquitin to or remove it from histones (**Figure 1**; **Table 1**).

HISTONE UBIQUITIN LIGASES

Polycomb group protein RING1B is the first identified ubiquitin ligase (E3) responsible for monoubiquitination of H2A at lysine 119 (Wang et al., 2004; Cao et al., 2005). Loss of RING1B dramatically decreases H2A monoubiquitination globally and at the promoters of specific genes (Wang et al., 2004; Cao et al., 2005). Two other RING domain containing proteins in the PRC1 (Polycomb Repressive Complex 1) complex, RING1A, and BMI1, strongly stimulate the E3 ubiquitin ligase activity of RING1B (Cao et al., 2005; Buchwald et al., 2006). Another H2A-specific

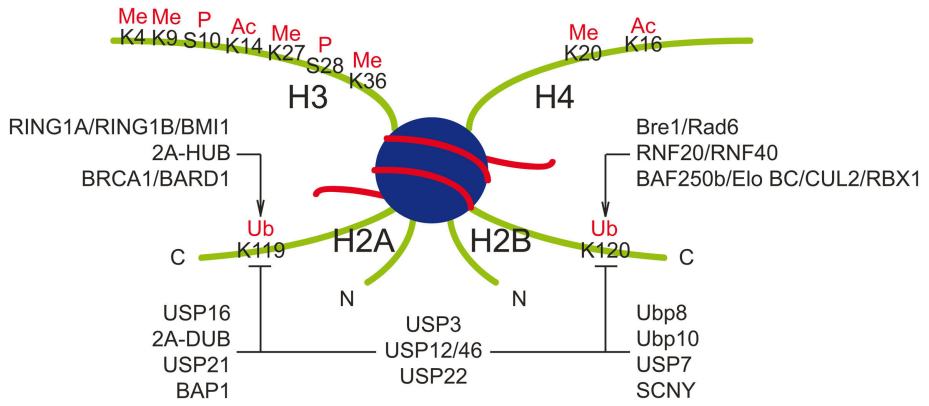

FIGURE 1 | Ubiquitin ligases and deubiquitinating enzymes responsible for monoubiquitination of histones H2A and H2B. Major post-transcriptional modifications on histone H3 and H4 tails are also shown. Ac, acetylation; Me, methylation; P, phosphorylation.

Table 1 | Functions of histone modifying enzymes for monoubiquitination.

Enzyme	Species	Histone specificity	Enzymatic activity	Role in transcription	Reference
RING1A/RING1B/BMI1	Human	H2A	E3	Repression	Cao et al. (2005); Gearhart et al. (2006)
2A-HUB	Human	H2A	E3	Repression	Zhou et al. (2008)
BRCA1/BARD1	Human	H2A	E3	Repression	Chen et al. (2002); Zhu et al. (2011)
UbcH5c	Human	H2A	E2	N/A	Chen et al. (2002)
Bre1	Yeast	H2B	E3	Activation	Robzyk et al. (2000); Kao et al. (2004)
Rad6	Yeast	H2B	E2	Activation	Robzyk et al. (2000); Kao et al. (2004)
RNF20/40	Human	H2B	E3	Activation	Kim et al. (2005); Prenzel et al. (2011)
RAD6A/B	Human	H2B	E2	Activation	Kim et al. (2005)
UbcH6	Human	H2B	E2	Activation	Zhu et al. (2005)
USP16	Human	H2A	DUB	Activation	Joo et al. (2007); Shanbhag et al. (2010)
USP21	Human	H2A	DUB	Activation	Nakagawa et al. (2008)
2A-DUB	Human	H2A	DUB	Activation	Zhu et al. (2007)
BAP1	Human *Drosophila*	H2A	DUB	Activation	Scheuermann et al. (2010)
Ubp8	Yeast	H2B	DUB	Activation	Henry et al. (2003); Daniel et al. (2004)
Ubp10	Yeast	H2B	DUB	Repression	Emre et al. (2005)
Ubp7	*Drosophila*	H2B	DUB	Repression	van der Knaap et al. (2005)
SCNY	*Drosophila*	H2B	DUB	Repression	Buszczak et al. (2009)
UBP12/46	*Xenopus*	H2A H2B	DUB	Activation	Joo et al. (2011)
USP3	Human	H2A H2B	DUB	N/A	Nicassio et al. (2007)
USP22	Human	H2A H2B	DUB	Activation	Zhang et al. (2008); Zhao et al. (2008)

E3, 2A-HUB/hRUL138 is recruited by the NCoR/HDAC1/3 complex and catalyzes H2A monoubiquitination at Lysine 119 (Zhou et al., 2008). Breast cancer type 1 susceptibility gene (BRCA1) is also a potential E3 ubiquitin ligase for histone H2A. In an *in vitro* ubiquitination assay, BRCA1 cooperates with ubiquitin-conjugating enzyme (E2) UbcH5c to catalyze monoubiquitination of H2A/H2AX (Chen et al., 2002). In an *in vivo* study, BRCA1 co-localizes with H2Aub on satellite DNA, and loss of BRCA1 results in the decrease of H2Aub occupation in these regions (Zhu et al., 2011). Another RING finger containing protein BARD1, which carries an enzyme dead mutation in its RING finger, binds to BRCA1 and enhances its E3 activity (Xia et al., 2003). Although several ubiquitin ligases can catalyze H2A monoubiquitination, loss of RING1A, RING1B, or BMI1 leads to global decrease of H2Aub, suggesting the RING1A/RING1B/BMI1 complex is the major E3 in mammalian cells (de Napoles et al., 2004; Wang et al., 2004; Cao et al., 2005; Stock et al., 2007).

The enzymes responsible for H2B monoubiquitination were first identified in yeast: Rad6 as a ubiquitin-conjugating enzyme and Bre1 as a ubiquitin ligase (Robzyk et al., 2000; Hwang et al., 2003; Wood et al., 2003; Kao et al., 2004). There are two homologs of Bre1 in mammalian cells: RNF20 and RNF40 (Koken et al., 1991; Kim et al., 2005). RNF20 and RNF40 form a complex *in vivo*, associate with yRad6 homologs hRAD6A and hRAD6B, or another ubiquitin-conjugating enzyme UbcH6, and catalyze monoubiquitination of H2B at lysine 120 *in vitro* (Kim et al., 2005, 2009; Zhu et al., 2005). Knockdown of RNF20 or RNF40 significantly reduces H2Bub globally in human cells (Kim et al., 2005; Zhu et al., 2005).

The SWI/SNF-A subunit BAF250b associates with Elongin B and C, CUL2, and RBX1 to form a canonical Elongin BC containing ubiquitin ligase complex similar to the VHL or E4orf6 complex (Kamura et al., 1999; Querido et al., 2001; Yan et al., 2004). This BAF250b complex can also catalyze H2B monoubiquitination (Li et al., 2010).

Histones H2A and H2AX can also be polyubiquitinated by a related ubiquitin ligase complex. RNF8 and RNF168 catalyze formation of K63-linked polyubiquitination chain in histones H2A and H2AX at the site of DNA damage during DNA damage repair (Stewart et al., 2009). Polyubiquitination of H2A and H2AX facilitates the accumulation of DNA repair proteins including 53BP1 and BRCA1 at the DNA damage foci (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Wang and Elledge, 2007; Doil et al., 2009; Stewart et al., 2009).

HISTONE DEUBIQUITINATING ENZYMES

Like other histone modifications, monoubiquitination of histones H2A and H2B is reversible. The ubiquitin modification can be removed by ubiquitin specific peptidases known as deubiquitinating enzymes (DUBs).

Several DUBs, including USP16, 2A-DUB, USP21, and BRCA1 associated protein 1 (BAP1) were identified as H2A-specific. USP16 catalyzes H2A deubiquitination *in vitro* and *in vivo*, and plays important roles in H2Aub mediated *HOX* gene silencing, X chromosome inactivation, cell cycle progression, and DNA damage repair (Joo et al., 2007; Shanbhag et al., 2010). 2A-DUB interacts with PCAF and is required for full activation of androgen receptor-dependent genes (Zhu et al., 2007). Another H2A-specific DUB, USP21, was identified as a regulator of liver regeneration by deubiquitination of H2Aub at the promoters of regeneration related genes (Nakagawa et al., 2008). The polycomb protein BAP1, was identified as an H2A-specific histone C-terminal hydrolase. BAP1 removes monoubiquitin from H2A but not H2B *in vivo* and *in vitro*. Loss of BAP1 significantly increases H2Aub level and abolishes the repression of *HOX* genes in flies (Scheuermann et al., 2010).

Ubp8 and Ubp10 were identified as histone H2B DUBs in yeast (Henry et al., 2003; Daniel et al., 2004; Emre et al., 2005), but they have very different functions. Ubp8 co-localizes with H3K4me3, while Ubp10 binds to H3K79me3 enriched sites, as well as telomere and rDNA locus (Emre et al., 2005; Schulze et al., 2011). Ubp8 catalyzes H2B deubiquitination *in vitro* and loss of Ubp8 increases the global level of H2Bub (Henry et al., 2003; Daniel et al., 2004), suggesting that Ubp8 is the major H2Bub DUB in yeast. Furthermore, as a component of the SAGA acetylation complex, Ubp8 is required for the transcription of SAGA-regulated genes (Henry et al., 2003). In addition, USP7 has been shown to catalyze H2B deubiquitination and mediate epigenetic silencing of homeotic genes (van der Knaap et al., 2005). *Drosophila* ubiquitin protease SCNY, a homolog of yeast Ubp10, deubiquitinates monoubiquitinated H2B *in vitro* (Buszczak et al., 2009). Loss of SCNY increases monoubiquitinated H2B in larvae (Buszczak et al., 2009). Interestingly, SCNY is required for maintaining multiple types of adult stem cells (Buszczak et al., 2009).

In addition to H2A or H2B specific DUBs, several DUBs display dual specificity toward both H2Aub and H2Bub, such as USP3, USP12, and USP46. USP3 is required for cell cycle progression and genome stability, while USP12 and USP46 regulate *Xenopus* development (Nicassio et al., 2007; Joo et al., 2011). The Ubp8 homolog USP22 is a subunit of coactivator acetyltransferase hSAGA complex. It is recruited to the promoters by activators to deubiquitinate H2A and H2B, and is required for transcription activation (Zhang et al., 2008; Zhao et al., 2008).

Multiple histone DUBs were identified, suggesting that they may have redundant functions or act in a context-dependent manner. Although their redundancy was not extensively investigated, current literature supports the notion that these DUBs have context-dependent functions in various processes. Their functions may also be dictated by their expression patterns in different tissues and stages during development.

FUNCTION OF HISTONE UBIQUITINATION

As histones are the most abundant ubiquitinated proteins, their ubiquitination plays critical roles in many processes in the nucleus, including transcription, maintenance of chromatin structure, and DNA repair.

TRANSCRIPTION

Monoubiquitination of H2A and H2B have been clearly implicated in transcriptional regulation. H2Aub occupation is more frequently correlated with gene silencing, while H2Bub is mostly associated with transcription activation.

H2A ubiquitin ligases were found in transcription repressor complexes, such as the PRC1, BCoR, E2F6.com-1, and 2A-HUB complexes (Ogawa et al., 2002; Wang et al., 2004; Cao et al., 2005; Gearhart et al., 2006; Zhou et al., 2008). RING1B mediated H2Aub is required for polycomb targeted gene silencing (Cao et al., 2005). Furthermore, in an *in vitro* assay, H2Aub represses transcriptional initiation and inhibits the formation of transcriptional active markers H3K4me2 and me3 (Nakagawa et al., 2008). The fact that H2A DUBs are usually required for genes activation (Joo et al., 2007; Zhu et al., 2007; Zhao et al., 2008) provides a second line of evidence for the gene silencing function of H2Aub.

In contrast, H2Bub occupation is strongly correlated with active gene expression in most cases, likely through multiple mechanisms, including promoting other active histone modifications and Pol II elongation. In a ChIP-on-Chip experiment, H2B monoubiquitination was found in the transcribed regions of highly expressed genes (Minsky et al., 2008). In yeast, monoubiquitinated H2B is required for the COMPASS complex for di- and tri-methylation of H3 at lysine 4 (Dover et al., 2002; Sun and Allis, 2002; Lee et al., 2007a), which are active markers for transcription (Klose et al., 2007; Blair et al., 2011). Loss of E2, E3, or the ubiquitination site (K123) within H2B inhibits H3K4 methylation by the COMPASS complex (Sun and Allis, 2002; Wood et al., 2003). In mammalian cells, E2 UbcH6 and E3 complex RNF20/RNF40 are recruited to transcriptionally active genes (Zhu et al., 2005). RNF20 also binds to transcription factors such as p53 directly and functions as a coactivator (Kim et al., 2005). Overexpression of RNF20 leads to elevated H2B monoubiquitination globally, which leads to subsequent increase of methylation at lysine 4 and 79 in H3, and stimulation of *HOX* gene expression (Zhu et al., 2005). Conversely, knockdown of RNF20 decreases endogenous H2Bub,

H3K4, and K79 methylation, and therefore transcription (Kim et al., 2005). Moreover, transcriptional regulation by RNF20 is dependent on its E3 ligase activity (Shema et al., 2011).

Recent work implicated the function of H2Bub in regulating transcriptional elongation, suggesting that it is also a mechanism by which H2Bub plays a positive role in gene expression. H2B ubiquitination is associated with elongating RNA Polymerase II, and is necessary for reassembly of nucleosomes and restoration of the chromatin structure during the transcription elongation, thus influencing the kinetic properties of elongating Pol II (Xiao et al., 2005). Using a reconstituted chromatin transcription system, Reinberg and colleagues showed that H2Bub stimulates elongation by Pol II through the chromatin by promoting the replacement of H2A/H2B dimers from the core nucleosomes (Pavri et al., 2006). A recent study showed that monoubiquitinated H2B cooperates with acetylated H4 to disrupt chromatin compaction, which leads to an open and accessible chromatin structure (Fierz et al., 2011). Furthermore, yeast mutants carrying defects in H2B ubiquitination pathway display transcription elongation defects (Xiao et al., 2005).

However, H2B monoubiquitination may also repress gene expression in some cases. H2B DUBs, for example, were found in some coactivator complexes and they are required for coactivator-dependent gene activation in both yeast and mammalian cells (Henry et al., 2003; Zhang et al., 2008; Zhao et al., 2008). One of these DUBs, Ubp8, promotes Pol II CTD phosphorylation, which is a mark of transcription elongation and is required for co-transcriptional mRNA processing (Wyce et al., 2007). Both E3s and DUBs of H2Bub showed a positive effect on gene activation, suggesting the ubiquitination and deubiquitination cycle of H2B is required for full gene induction (Henry et al., 2003; Wyce et al., 2007).

Many studies demonstrated that histone ubiquitination and other histone modifications are inter-connected and they act in combination and/or sequentially to regulate transcription. For example, H2B monoubiquitination is required for both H3K4 methylation and H3K79 methylation (Dover et al., 2002; Sun and Allis, 2002; Lee et al., 2007a). H3K27 demethylase UTX was shown to suppress the recruitment of PRC1 and subsequent H2A monoubiquitination (Lee et al., 2007b). It was also shown that H2A monoubiquitination is coupled to H3K36me2 demethylation (Lagarou et al., 2008). These findings indicate that precise transcriptional control requires the concerted actions of multiple histone modifications.

DNA DAMAGE RESPONSE

Current studies suggest that histone ubiquitination is a general histone modification induced by DNA damage and plays important roles in DNA damage response. DNA damage has emerged as a major culprit in cancer and many other diseases. Inherited impairments in DNA repair usually leads to a higher risk of cancer. Cells developed a defense system against DNA damage, called DNA damage response, which includes recruitment of DNA repair machinery, cell cycle arrest, and lesion tolerance or apoptosis (Hoeijmakers, 2001).

One of the classic models to trigger DNA damage response is to introduce DNA double-strand break (DSB). Following DSBs, the histone variant H2AX is rapidly phosphorylated at the γ position (γH2AX) along chromatin tracks flanking DSBs by ATM, ATR, and DNA-PK (Falck et al., 2005). H2AX phosphorylation facilitates the accumulation of DNA damage response regulators, Mdc1/NFBD1(Stewart et al., 2003; Xu and Stern, 2003), RNF8 and RNF168. RNF8 and RNF168 catalyze the K63-linked polyubiquitination chain formation on histone H2A and H2AX (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Stewart et al., 2009). K63-linked polyubiquitinated histones provide a recognition element that recruits RAP80 through its ubiquitin interaction motif (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). The subsequent recruitment of BRCA1 and the intact IR induced G2/M checkpoint are dependent on RAP80 and its ubiquitin binding motif that recognizes K63-linked polyubiquitin chains on H2A and H2AX (Kim et al., 2007; Sobhian et al., 2007; Wang and Elledge, 2007; Wang et al., 2007). Consistent with these findings, knockdown of histone ubiquitination enzymes impairs DSB-associated polyubiquitination of H2A and H2AX, inhibits retention of 53BP1 and BRCA1 at the DSB sites, and sensitizes cells to ionizing radiation (Huen et al., 2007; Kolas et al., 2007; Mailand et al., 2007; Stewart et al., 2009).

In addition to polyubiquitination, monoubiquitination of histones H2A, H2B, and H2AX also occurs at the sites of DNA damage. RING1B/BMI1 and RNF20/RNF40 are recruited to DSB site and catalyze H2A/H2AX monoubiquitination at lysine 119 and H2B monoubiquitination at lysine 120, respectively (Bergink et al., 2006; Marteijn et al., 2009; Wu et al., 2009, 2011; Ginjala et al., 2011; Moyal et al., 2011; Pan et al., 2011). Furthermore, depletion of RNF20 or interference with histone H2B monoubiquitination disrupts the recruitment of DNA repair machine proteins in both non-homologous end joining (NHEJ) and homologous recombination repair (HRR) pathways to the DSB (Moyal et al., 2011). Histone DUBs, such as USP3 and K63-ub DUB BRCC36, are also critical for efficient DNA repair, suggesting that a dynamic regulation of histone ubiquitination and deubiquitination is required for DNA damage response (Shao et al., 2009). It was proposed that monoubiquitination of histones H2A and H2B interferes with chromatin compaction and therefore facilitates assembly of the repair machinery on the DNA damage foci (Moyal et al., 2011), but the mechanisms of action remain elusive.

OTHER FUNCTIONS

H2A monoubiquitination occurs on the inactive X chromosome in female mammals (de Napoles et al., 2004; Fang et al., 2004). Both RING1B and H2Aub are involved in the initiation of imprinted and random X chromosome inactivation (de Napoles et al., 2004; Fang et al., 2004).

In addition to transcription regulation, monoubiquitinated H2B is required for chromatin function in other ways. H2Bub is required for chromatin boundary integrity, and loss of H2Bub leads to the spreading of other histone modifications (Ma et al., 2011). The present of H2Bub also interferes with chromatin compaction and results in an open chromatin structure (Fierz et al., 2011). H2Bub is also shown to play an important role in homologous recombination through chromatin remodeling by recruiting chromatin remodeling factors. Cells lacking RNF20 or expressing H2B K120R, which lacks ubiquitin conjugation site, exhibit defects in HRR (Nakamura et al., 2011).

Histone ubiquitination and deubiquitination play essential roles in stem cell maintenance and differentiation, likely through controlling the expression of key pluripotency and differentiation genes. BMI1 is required for the self-renewal and maintenance of hematopoietic stem cells (Lessard and Sauvageau, 2003; Park et al., 2003) and neural stem cells (Molofsky et al., 2003). In flies, H2B DUB SCNY is required for the maintenance of germline stem cells, follicle stem cells, and intestinal stem cells. SCNY mutant animals display reduced number and half-life of germline stem cells (Buszczak et al., 2009). In mouse embryonic stem cells, ubiquitinated H2A restrains poised RNA polymerase II at a subset of developmental regulator genes. Loss of RING1A and RING1B releases poised RNA polymerase II and subsequent gene de-repression (Stock et al., 2007).

Furthermore, a recent landmark paper showed that H2Bub can function in trans independent of transcription (Latham et al., 2011). During mitosis, H2Bub is required for the kinetochore protein Dam1 methylation (Latham et al., 2011). Depletion of E2, E3 for H2B monoubiquitination or mutation of Lys-123 in H2B inhibits methylation of Dam1 (Latham et al., 2011). These results suggest that H2Bub also plays important roles outside of chromatin and is required for chromosome segregation.

HISTONE UBIQUITINATION AND CANCER

Histone modifications play critical roles in genes expression and DNA repair. Aberrations of these processes often cause cancers (Jones and Baylin, 2007). Many histone modifying enzymes have been identified as oncogene or tumor suppressors (Chandrasekharappa et al., 1997; Yokoyama et al., 2004; Wissmann et al., 2007; Lin et al., 2011). Therefore, it is not a surprise that more and more connections between histone ubiquitination and cancer have been discovered. Monoubiquitinated histone H2A and H2B were found to be dramatically down-regulated in prostate and breast tumors, respectively (Zhu et al., 2007; Prenzel et al., 2011). Recently advances have also linked the writers, erasers, and readers of histone ubiquitination to tumorigenesis.

BRCA1

Inactivation of tumor suppressor BRCA1 leads to breast and ovarian cancer. Female individuals carrying a mutated *BRCA1* allele have an estimated risk of 87% for breast cancer and 44% for ovarian cancer by age 70 (Ford et al., 1994). BRCA1 associated protein BAP1 is also a tumor suppressor in multiple cancers, including lung cancer, breast cancer, uveal melanoma, and mesothelioma (Harbour et al., 2010; Testa et al., 2011; Wiesner et al., 2011).

The BRCA1 protein is a RING finger domain containing E3 ubiquitin ligase. H2A and H2B were identified as BRCA1 substrates in an *in vitro* assay (Chen et al., 2002; Mallery et al., 2002). A more recently report showed that BRCA1 binds to satellite DNA regions and catalyzes monoubiquitination of H2A *in vivo* (Zhu et al., 2011). Loss of BRCA1 is associated with loss of H2A ubiquitination at satellite repeats and de-repression of satellite transcription. Cells lacking BRCA1 are impaired in organization of heterochromatin structure. More interestingly, ectopic expression of H2A fused to ubiquitin, which is a mimic of natural monoubiquitinated H2A, rescues BRCA1 phenotypes (Zhu et al., 2011). These findings indicate that BRCA1 maintains heterochromatin structure via monoubiquitination of H2A. Furthermore, the fact that satellite DNA transcripts are increased in BRCA1 mutant breast cancer samples, and the relationship between monoubiquitination of H2A and satellite DNA repression suggest that dysregulation of H2Aub plays important roles in tumorigenesis.

RNF20 AND RNF40

RNF20 is the major H2B specific E3 ubiquitin ligase in mammalian cells. Besides a significant decrease in the cellular pool of monoubiquitinated H2B, RNF20 depletion causes increased expression of some proto-oncogenes and growth-related genes including c-myc and c-Fos (Shema et al., 2008). Further study discovered that RNF20 represses gene expression by disrupting the interaction between TFIIS and PAF1 elongation complex and inhibiting transcriptional elongation. Those effects are also dependent on the E3 ligase activity of RNF20 (Shema et al., 2011). In addition, RNF20 depleted cells showed decreased expression of the p53 tumor suppressor, and increased cell migration and tumorigenesis. Moreover, hypermethylation of RNF20 promoter was observed in tumor samples (Shema et al., 2008). These findings suggest that H2B ubiquitin ligase RNF20 is a putative tumor suppressor. Furthermore, the RNF40, a binding partner of RNF20 and another major E3 for H2B monoubiquitination also showed tumor suppressive activity in breast cancer cells (Prenzel et al., 2011).

USP22

USP22 is a ubiquitin hydrolase and catalyzes the removal of ubiquitin from monoubiquitinated histones H2A and H2B (Zhang et al., 2008, 2011; Zhao et al., 2008). USP22 is a putative cancer stem cell marker and was found to be highly expressed in malignant tumor samples. High level of USP22 in tumor tissues is associated with poor clinical outcome, including high risk of recurrence, metastasis, and resistance to chemotherapy (Glinsky, 2005; Glinsky et al., 2005; Zhang et al., 2011). Further studies determined that USP22 is recruited to the gene promoters by Myc and is required for the activation of Myc target genes (Zhang et al., 2008). Depletion of USP22 compromises Myc functions, including transformation. USP22 also play roles in cell cycle regulation, where depletion of USP22 increases the expression of p53 and p21, inhibits proliferation, and induces cell cycle arrest at G1 phase (Zhang et al., 2008; Lv et al., 2011).

CONCLUSION

It is now well established that histone modifications and enzymes catalyzing their addition or removal are essential for normal cellular functions. Rapid advances of this field revealed that fine tuning of histone ubiquitination and deubiquitination is required for gene expression, DNA repair, and many other biological processes. Therefore aberrations of histone ubiquitination or deubiquitination lead to multiple human diseases including cancer. However, the precise mechanisms by which histone ubiquitination contributes to these biological processes are still poorly understood and require further investigation. Related essential questions include the crosstalk between histone ubiquitination and DNA methylation or other chromatin marks. Future studies that help decipher the essential roles of epigenetic regulation by histone

ubiquitination in cellular homeostasis and pathological conditions like cancer will definitely benefit the identification of "druggable" targets for personalized cancer therapies.

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