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Review

The emerging family of CULLIN3-RING ubiquitin ligases (CRL3s): cellular functions and disease implications

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Protein ubiquitylation is a post-translational modification that controls all aspects of eukaryotic cell functionality, and its defective regulation is manifested in various human diseases. The ubiquitylation process requires a set of enzymes, of which the ubiquitin ligases (E3s) are the substrate recognition components. Modular CULLIN-RING ubiquitin ligases (CRLs) are the most prevalent class of E3s, comprising hundreds of distinct CRL complexes with the potential to recruit as many and even more protein substrates. Best understood at both structural and functional levels are CRL1 or SCF (SKP1/CUL1/F-box protein) complexes, representing the founding member of this class of multimeric E3s. Another CRL subfamily, called CRL3, is composed of the molecular scaffold CULLIN3 and the RING protein RBX1, in combination with one of numerous BTB domain proteins acting as substrate adaptors. Recent work has firmly established CRL3s as major regulators of different cellular and developmental processes as well as stress responses in both metazoans and higher plants. In humans, functional alterations of CRL3s have been associated with various pathologies, including metabolic disorders, muscle, and nerve degeneration, as well as cancer. In this review, we summarize recent discoveries on the function of CRL3s in both metazoans and plants, and discuss their mode of regulation and specificities.

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Introduction

Regulation of protein stability by the ubiquitin/proteasome system (UPS) participates in a broad range of physiologically and developmentally controlled processes in all eukaryotes (Ciechanover *et al*, 2000; Smalle and Vierstra, 2004). A critical step in this pathway involves ubiquitin ligases (also known as E3 enzymes or E3s), which facilitate the transfer of ubiquitin moieties to substrate proteins, as a preparative step for their degradation by the 26S proteasome. Several hundred different E3s have been identified in metazoan and plant genomes, based on specific, commonly shared structural motifs. Among them, CULLIN-RING ubiquitin ligases (CRLs) are the most prevalent class (Petroski and Deshaies, 2005; Hua and Vierstra, 2011). CRLs are multimeric E3s, in which one particular CULLIN protein serves as a molecular scaffold linking up the catalytic module, composed of a RING finger domain protein and a ubiquitin-conjugating (or E2) enzyme, to a specific substrate recognition module, which physically interacts with target proteins. Among the CRL family, the founding member is the SCF (SKP1/CUL1/F-box protein (FBP)) complex (Figure 1A), which employs one of 68 (human) or 700 (*Arabidopsis thaliana*) FBPs for substrate recognition (Gagne *et al*, 2002; Jin *et al*, 2004). Beside CUL1, eukaryotic genomes encode additional cullins (CUL2, CUL3, CUL4, CUL5, and CUL7) (Gieffers *et al*, 2000; Sarikas *et al*, 2008) that have likewise been found to form protein complexes with E3 activities, modifying a variety of substrates by using distinct sets of adaptor modules.

Recent research has firmly established CUL3 as the molecular scaffold of a major class of CRLs controlling different developmental and stress responses (Table I) as well as human pathologies (Table II). CUL3 is a highly conserved CULLIN family member present in the genomes of all eukaryotes. In *C. elegans*, *CUL3* loss-of-function leads to a defect of cytokinesis in single-cell embryos (Kurz *et al*, 2002), and the deletion of this gene in mouse produces an arrest during early embryogenesis (Singer *et al*, 1999). In the model plant *Arabidopsis thaliana*, disruption of the two related *CUL3A* and *CUL3B* genes also causes embryo lethality, affecting both embryo pattern formation and endosperm development (Figueroa *et al*, 2005; Thomann *et al*, 2005; Gingerich *et al*, 2007). In contrast to this situation in multicellular organisms, the function of *CUL3* orthologues is not essential in either budding or fission yeasts (Geyer *et al*, 2003; Michel *et al*, 2003).

At the structural level, CUL3 interacts with BTB/POZ (for '*B*ric-a-brac, *T*ramtrack and *B*road Complex/*P*ox virus and *Z*inc finger', hereafter referred to simply as BTB) domain proteins, which function as substrate-specific adaptors (Furukawa *et al*, 2003; Xu *et al*, 2003; Pintard *et al*, 2003b). They bind CUL3 via the BTB domain, and commonly direct substrate specificity through an independent additional protein–protein interaction domain (PID) (Figure 1A),

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Glossary

ABA	abscisic acid
adRP	autosomal dominant Retinitis Pigmentosa
BTB/POZ	Bric-a-brac, Tramtrack and Broad Complex/Pox virus and Zinc finger
CAND1	Cullin-Associated and Neddylation-Disassociated 1
Ci	Cubitus Interruptus
CPC	Chromosomal Passenger Complex
CRL	CULLIN-RING Ubiquitin Ligase
DCN1	Defective in Cullin Neddylation 1
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
FBP	F-box protein
IFN	Interferon
MATH	Meprin and TRAF homology
NAE	Nedd8-Activating Enzyme
NPH3	Nonphototropic Hypocotyl 3
Nrf2	NF-E2-related factor 2
PID	protein interaction domain
PLK1	Polo-Like Kinase 1
SA	Salicylic Acid
SAC	Spindle Assembly Checkpoint
SAR	Systemic Acquired Resistance
SCF	SKP1/CUL1/F-box protein
TPR	Tetratrico Peptide Repeat
UPS	Ubiquitin/Proteasome System

thus uniting the functions of the SKP1/FBP heterodimer in SCF/CRL1 complexes in a single polypeptide. Sequence analyses have so far identified over a dozen different protein domains that are associated, sometimes in combinations, with the BTB domain (Stogios *et al*, 2005). Some of these are widely distributed throughout eukaryotic genomes (such as the Meprin and TRAF homology (MATH) domain), while others are specific to either metazoans (e.g., the Kelch domain) or plants (e.g., the BTB-non-phototropic hypocotyl 3 (NPH3) domain; Figure 1B). It should be noted that only a subset of all BTB domain proteins actually serve as CRL3 adaptors, and they are set apart from the large fraction of zinc-finger BTB proteins by the presence of an additional paired helical structure (called 3-box motif) positioned C-terminal to the BTB domain, which fulfils an important function in CRL3s assembly analogous to the F-box and SOCS box motifs of other Cullin-based E3s (Zhuang *et al*, 2009).

It is noteworthy that the number of BTB proteins—and thus potential CRL3s—varies a lot between organisms. The human genome encodes nearly 200 BTB domain proteins, (Stogios *et al*, 2005), although those lacking the 3-box structures may not be engaged in functional CRL3 complexes, while about 80 BTB proteins have been identified in *A. thaliana* (Dieterle *et al*, 2005; Figueroa *et al*, 2005; Gingerich *et al*, 2005) and even fewer in *D. melanogaster*. This contrasts the situation for the SCF/CRL1 complexes, where the large number of FBPs in plants indicates increased versatility (Gagne *et al*, 2002). However, as will be illustrated below, recent research indicates that this does not mean that CRL3s are of minor importance in plants.

Biological processes involving CRL3s in metazoans

A key regulator of basic cellular functions in metazoans

The ubiquitin/proteasome system is a major regulator of the cell cycle in all eukaryotes, targeting dozens of regulatory proteins for degradation and thus ensuring irreversible

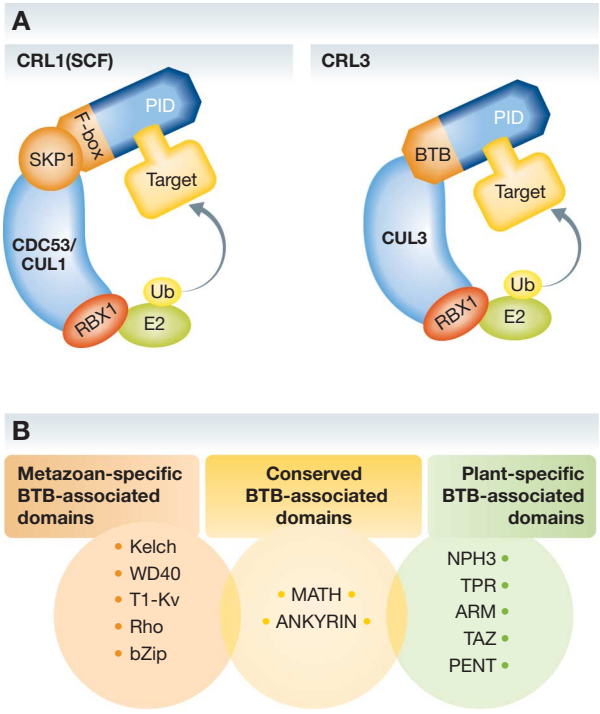

Figure 1 Structural organization of SCF/CRL1 and the CRL3 complexes. (**A**) The SCF/CRL1 and the CRL3 complexes share a similar catalytic core module composed of the scaffold proteins CUL1 and CUL3, respectively, and the RING finger protein RBX1 (also known as Hrt1 or ROC1). Single-subunit BTB domain proteins bridge CUL3-RBX1 to substrates, while this function requires an SKP1/FBP heterodimer in SCF/CRL1. Substrate recognition is governed by an independent protein–protein interaction domain (PID) found in most of the FBPs and CUL3-interacting BTB domain proteins. (**B**) Non-exhaustive list of protein domains is commonly found associated with the BTB domain in CRL3 adaptors. MATH and Ankyrin domains occur in both metazoans and higher plants, while other domains are specific to either kingdom. BTB-KELCH; BTB-WD40; BTB-T1-Kv (voltage-gated potassium channel T1); BTB-Rho (Ras homology); BTB-bZip (basic leucine Zipper); BTB-MATH (Meprin and TRAF homology); BTB-ANKYRIN repeat; BTB-NPH3 (non-phototropic hypocotyl 3); BTB-TPR (Tetratrico Peptide Repeat); BTB-ARM (Armadillo); BTB-TAZ (Transcriptional Adaptor Zinc finger); BTB-PENT (Pentapeptide).

cell-cycle stage transitions (Mocciaro and Rape, 2012). While the key E3s for this are the anaphase promoting complex/cyclosome (APC/C) and SCF complexes, more recently CRL3s also entered into the game. In mammalian cells, CRL3s play an essential function in the progression of mitosis and completion of cytokinesis via ubiquitination of Aurora B kinase and thereby preventing chromosomal passenger complex (CPC) accumulation on mitotic chromosomes (Sumara *et al*, 2007). Aurora B is poly-ubiquitylated on mitotic chromosomes during prometaphase, in a manner dependent on the Kelch-BTB proteins KLHL9 and KLHL13. Rather than triggering its degradation by the proteasome, Aurora B polyubiquitylation during mitosis however seems to serve as a signal for its extraction from chromosomes. During anaphase, another BTB protein, KLHL21, was shown to mono-ubiquitylate Aurora B on microtubules of the spindle midzone (Maerki *et al*, 2009). Similarly, a CUL3-KLHL22 E3 ligase complex mono-ubiquitylates Polo-like kinase 1 (PLK1) to remove it from kinetochores after chromosomes have achieved bi-orientiation in metaphase (Beck *et al*, 2013), with this non-degradative PLK1 ubiquitylation being

Table I List of functional CUL3-based ubiquitin ligases and their substrates in different organisms

Organisms	Name	Protein domains	Substrates	Function	Reference
Mammals	KLHL9/13/21	BTB-Kelch	Aurora B[a]	Mitotic progression	Sumara *et al* (2007) and Maerki *et al* (2009)
	KLHDC5	BTB-Kelch	p60/katanin	Mitotic spindle formation	Cummings *et al* (2009)
	BACURD	BTB[b]	RhoA	Actin cytoskeleton structure	Chen *et al* (2009b)
	KLHL12	BTB-Kelch	Dsh	Wnt/β-catenin signalling	Angers *et al* (2006)
			Sec31[a]	Collagen secretion	Jin *et al* (2012)
	KEAP1/KLHL19	BTB-Kelch	Nrf2	Oxidative stress response	Cullinan *et al* (2004), Kobayashi *et al* (2004) and Zhang *et al* (2004)
			IKKβ	NF-κB signalling	Lee *et al* (2009)
	KLHL20	BTB-Kelch	PML	Hypoxia response	Yuan *et al* (2011)
			DAPK	IFN-induced cell death	Lee *et al* (2010)
	KLHL22	BTB-Kelch	PLK1[a]	Chromosome segregation	Beck *et al* (2013)
	KLHL25	BTB-Kelch	4E-BP1	Translational homeostasis	Yanagiya *et al* (2012)
	KLHL8	BTB-Kelch	Rapsyn	Neurotransmitter signalling	Nam *et al* (2009)
	KLHL3	BTB-Kelch	WNK1, WNK4	Blood pressure regulation	Ohta *et al* (2013) and Wakabayashi *et al* (2013)
	SPOP	MATH-BTB	Gli2/Gli3	Hedgehog signalling	Chen *et al* (2009a)
			Daxx	Transcription, apoptosis	Kwon *et al* (2006)
			SRC-3	Transcription by nuclear receptors	Li *et al* (2011)
			PIPKIIβ[a]	Phosphoinositide signalling	Bunce *et al* (2008)
			BMI1[a] MacroH2A[a]	Epigenetic silencing	Hernandez-Munoz *et al* (2005)
D. rerio	Btbd6a	BTB-PHR	Plzf	Neurogenesis	Sobieszczuk *et al* (2010)
D. melanogaster	HIB/SPOP	MATH-BTB	Ci/Gli	Hedgehog signalling	Zhang *et al* (2006)
			Puckered	TNF-mediated JNK signalling	Liu *et al* (2009)
C. elegans	KEL-8	BTB-Kelch	RPY-1	Neurotransmitter signalling	Schaefer and Rongo (2006) and Nam *et al* (2009)
	MEL-26	MATH-BTB	MEI-1	Microtubule reorganization	Pintard *et al* (2003b)
A. thaliana	ETO1/EOL1/EOL2	BTB-TPR	Type-2 ACSs	Ethylene biosynthesis	Christians *et al* (2009), Wang *et al* (2004) and Thomann *et al* (2009)
	NPH3	BTB-NPH3	PHOT1	Phototropism	Roberts *et al* (2011)
	NPR3/NPR4	BTB-Ank-repeat	NPR1	Systemic acquired resistance (SAR)	Fu *et al* (2012)
	BPM1-6	MATH-BTB	AtHB6	ABA response	Lechner *et al* (2011)
			WRI1	Fatty acid metabolism	Chen *et al* (2013)

BTB-associated domains and substrates supported by strong *in vivo* evidence are also given.
[a]Substrates that may not be degraded and whose ubiquitylation may serve non-proteolytic functions.
[b]BACURD contains 180 C-terminal residues with no recognizable sequence motif.

necessary for spindle assembly checkpoint (SAC) silencing and mitotic chromosome segregation. Thus, CUL3 recruits various BTB-containing proteins to target cell-cycle kinases, and possibly also other cell-cycle regulators, at distinct subcellular localizations and at different steps of mitosis. Moreover, the fact that CRL3s not only target substrates for proteasomal degradation, but also reversibly regulate processes by controlling subcellular localization and possibly even the activity and/or interactions of substrates in space and time, significantly expands the versatility and potential roles of CRL3s.

One of the first identified CRL3 substrate adaptors is the well-characterized nematode MATH-BTB protein MEL-26 (reviewed in Pintard *et al*, 2004). MEL-26 recruits the microtubule-severing katanin protein MEI-1, which is required for meiotic spindle formation but thereafter undergoes rapid CRL3-dependent degradation prior to the onset of mitotic divisions, when its persistence would lead to small and misoriented mitotic spindles. This mechanism appears to be conserved in metazoans, as the mammalian katanin catalytic subunit is also degraded via CRL3-mediated ubiquitylation, involving the Kelch repeat-containing BTB adaptor protein KLHDC5 (Cummings *et al*, 2009).

In addition to microtubule dynamics, CRL3 regulation also affects the actin cytoskeleton (Chen *et al*, 2009b). Here, the BTB protein BACURD, which does not contain a known recognizable substrate recognition motif in its C-terminal region, mediates the turnover of the small GTPase RhoA that controls the organization of actin cytoskeleton structure. Failure to degrade RhoA leads to abnormal stress fibres and inhibits the migration capabilities of mammalian cells.

Protein trafficking pathways

CUL3 and its BTB adaptor protein KLHL12 are important regulators of embryonic stem (ES) cell morphology, by affecting the deposition of the extracellular matrix component collagen, which is essential in all metazoans and important for ES cell division (Jin *et al*, 2012). Similarly to KLHL22, KLHL12 promotes mono-ubiquitylation of its target SEC31, a coat protein of COPII vesicles, and this allows the formation of enlarged COPII vesicle structures required for exocytosis and deposition of rigid, rod-shaped collagen molecules. In addition to secretion, CUL3 has also been implicated in the regulation of late endosome maturation, although the

Table II List of mutations detected in CUL3 and BTB substrate-specific adaptors in patients suffering from indicated diseases

Name	Disease	Mutation	Domain	Effect	Reference
CUL3	Pseudohypoaldosteronism type II (PHAII)/Gordon's syndrome/ hypertension	Delection aa 403–459	Segment between BTB-binding and RING binding domains	Loss of KLHL3 binding	Boyden *et al* (2012) and Wakabayashi *et al* (2013)
KLHL3	PHAII/Gordon's syndrome/hypertension	Numerous recessive and dominant mutations[a] A77E M78V E85A C164F Q309R R384Q L387P S410L S432N R528H R528C N529K	BTB BACK Kelch	Loss of CUL3 binding Loss of CUL3 binding Loss of substrate binding	Boyden *et al* (2012), Louis-Dit-Picard *et al* (2012), Ohta *et al* (2013) and Wakabayashi *et al* (2013)
KLHL7	Autosomal dominant Retinitis Pigmentosa (adRP)/Blindness	A153T A153V	BACK	Loss of CUL3 binding Lower E3 ligase activity	Kigoshi *et al* (2011)
KLHL9	Distal myopathy/skeletal muscle atrophy	L95F	BTB	Reduction in CUL3 binding	Cirak *et al* (2010)
KBTBD13	Nemaline myopathy (NEM)	R248S[a] K390N[a] R408C[a]	Kelch	Predicted disruption of β propeller structure	Sambuughin *et al* (2012)
Gigaxonin	Giant axonal neuropathy GAN/neuropathy of peripheral nerves and central nervous system	R15S[a] S52G[a] S79L[a] V82F[a] R138H[a] R269Q[a] R293X L309R[a] C393X[a] W401X[a] Q483X[a] E486K[a] R545C[a] C570Y[a]	BTB BACK Kelch	Predicted loss of CUL3 binding Loss of substrate binding	Bomont *et al* (2000) and Ding *et al* (2002)
KCTD7	Epilepsy, progressive myoclonic 3 (EPM3) Neuronal ceroid lipofuscinosis (NCL)	R99X[a] R184C	BTB	Protein truncation Loss of CUL3 binding	Van Bogaert *et al* (2007) and Staropoli *et al* (2012)
Keap1	Lung cancer	R272C G333S G364C L413R R415G G430C	BACK Kelch	Inhibition of E3 ligase activity but not binding to CUL3 Loss of substrate binding	Padmanabhan *et al* (2006), Singh *et al* (2006) and Ohta *et al* (2008)

[a]Detected mutations, of which predicted effects were not confirmed experimentally.

BTB adaptor proteins and their substrates involved in this process remain to be identified (Huotari *et al*, 2012).

Transcription in developmental signalling

In *Drosophila*, morphogens such as Hedgehog (Hh) have key roles in developmental processes. A pivotal mediator of Hh signalling, the transcription factor Cubitus Interruptus (Ci), needs to be specifically expressed in and sometimes restricted to specific tissues during development. One way to achieve this is targeted proteolysis, as illustrated by the MATH-BTB protein HIB/SPOP, which is expressed in the *Drosophila* eye disc posterior to the morphogenic furrow and that promotes Ci degradation to ensure normal eye development (Zhang *et al*, 2006). This process appears to be conserved in metazoans, as the mammalian SPOP homologue serves as a CRL3 adaptor for degradation of Gli2 and Gli3, two Gli transcription factors homologous to *Drosophila* Ci (Chen *et al*, 2009a). Importantly, the work on SPOP CRL3s defines mechanistically how SPOP interacts with its substrates to control transcriptional outputs (Chen *et al*, 2009a; Zhuang *et al*, 2009).

In vertebrates, another important signalling protein targeted by a CRL3 complex is Dishevelled (Dsh) (Angers *et al*, 2006), which constitutes a critical node in cell differentiation/proliferation decisions via the Wnt/β-catenin signalling pathway. Therefore, Dsh protein levels need to be

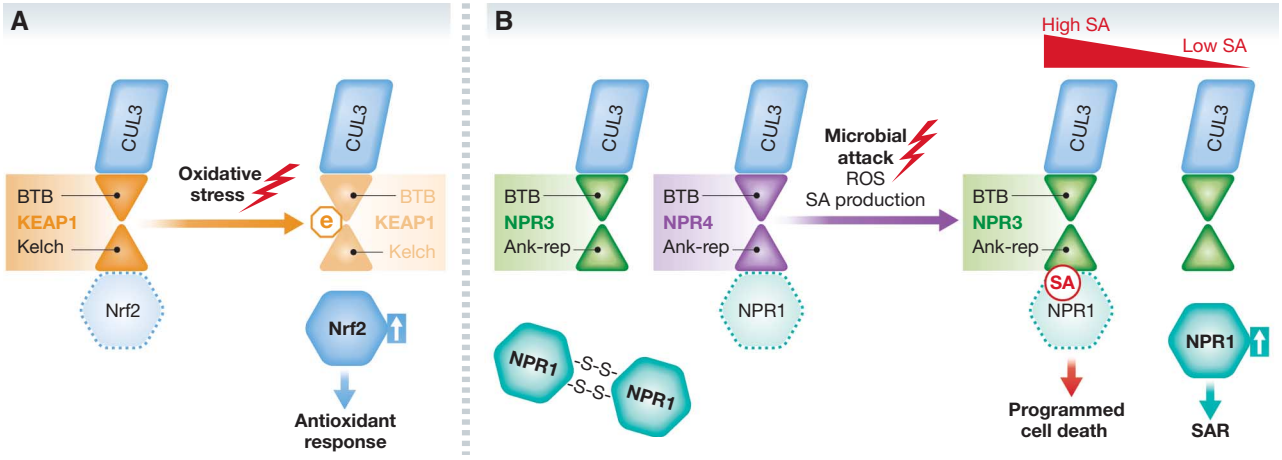

Figure 2 Mode of regulation of CRL3 activity and substrate recognition. (**A**) Nrf2 is constitutively targeted for Keap1-dependent degradation under normal conditions. In response to oxidative stress, oxidative modifications (denoted as (e), electrophile) on Keap1 impair its activity and result in Nrf2 stabilization. (**B**) In plant immunity, the transcription coactivator NPR1 is regulated at several levels. In unchallenged cells, NPR1 is predominantly sequestered in the cytoplasm in an oligomeric form through redox-sensitive intermolecular disulphide bonds. Upon pathogen infection, salicylic acid (SA) signals lead to alterations in reduction potential and partially relieves NPR1 to enter the nucleus. High SA concentrations immediately at sites of infection promote its binding to the BTB protein NPR3 and enhance NPR3–NPR1 interaction and subsequent NPR1 degradation, thereby favouring programmed cell death. Lower SA levels in neighbouring cells are insufficient to trigger NPR3-mediated NPR1 ubiquitylation, enabling NPR1 to accumulate and establish systemic acquired resistance (SAR). See text for details.

tightly regulated for normal embryonic development, and this is achieved through Wnt signal-dependent Dsh interaction with the BTB-Kelch protein KLHL12, leading to Dsh degradation.

Transcription in stress responses

One of the best-understood CRL3 roles in mammalian cells lies in the Keap1-Nrf2 (NF-E2-related factor 2) stress response pathway (for a recent in-depth review, see Taguchi *et al*, 2011). Nrf2 is a major transcriptional activator that induces expression of numerous protective genes in response to oxidative stress. Under normal growth conditions (i.e., in the absence of cellular stress), the BTB-Kelch substrate adaptor Keap1 triggers Nrf2 ubiquitin-dependent degradation by the proteasome in the cytoplasm (Cullinan *et al*, 2004; Kobayashi *et al*, 2004; Zhang *et al*, 2004). However, several cysteine residues in Keap1 can react with electrophiles produced during stress, which negatively affects CUL3-Keap1 ubiquitin E3 ligase activity (Dinkova-Kostova *et al*, 2002; Wakabayashi *et al*, 2004) (Figure 2). Upon oxidative stress, Nrf2 is therefore free to translocate into the nucleus and bind to the anti-oxidant responsive elements (AREs) in the promoter regions of its target genes. Besides Nrf2, Keap1 also recognizes other target proteins, such as the oncogenic kinase IKKβ (Lee *et al*, 2009) (discussed in more detail below).

Other levels of gene expression control

CRL3s can also control gene expression at other levels than transcriptional activation. In mammalian cells, the CRL3 adaptor SPOP mediates ubiquitylation of the Polycomb group protein BMI1 and the variant histone MacroH2A1 (Hernandez-Munoz *et al*, 2005), apparently affecting their function in a non-proteolytic fashion. CRL3-SPOP function is required for proper MacroH2A1 localization to the inactive X chromosome, and might thus be actively involved in the epigenetic silencing process that leads to X inactivation.

Further downstream in the process of gene expression, CRL3 was recently implicated in the control of translational homeostasis in mammals (Yanagiya *et al*, 2012). Here, the BTB adaptor KLHL25 promotes degradation of 4E-BP1, a protein that acts as a repressor of translation initiation. 4E-BP1 is only targeted when it is hypophosphorylated and therefore unable to interact with the mRNA cap-binding protein eIF4E, providing a means to control the levels of translation to maintain cellular homeostasis.

Cell death

In mammalian cells, CUL3 and the adaptor KLHL20 ubiquitylate the death-associated protein kinase (DAPK), an apoptosis mediator involved in interferon (IFN)-induced cell death as well as in response to a variety of other stimuli (Lee *et al*, 2010). Interestingly, this process is controlled at the level of sequestration of the CRL3 adaptor, whereby IFN induction leads to KLHL20 sequestration in promyelocytic leukaemia (PML) nuclear bodies, thus disrupting its interaction with DAPK and stabilizing the kinase (Lee *et al*, 2010).

The MATH-BTB protein SPOP is also involved in various apoptotic pathways. In *Drosophila*, SPOP mediates degradation of the Jun kinase phosphatase Puckered (Puc), which is required for apoptosis depending on the tumour necrosis factor (TNF) Eiger during embryonic segmentation (Liu *et al*, 2009). Human SPOP is involved in the turnover of the death-associated protein DAXX, an anti-apoptotic regulator (Kwon *et al*, 2006).

An unexpected mechanism of CUL3 action is exemplified by its role in vertebrate caspase activation (Jin *et al*, 2009). CUL3-dependent ubiquitylation of caspase-8 does not lead to its degradation, but instead promotes its stabilization and thus apoptosis induction. Moreover, CUL3 directly associates with caspase-8 and may not require a BTB-domain adaptor protein, although the presence of an as-yet unidentified copurified adaptor protein cannot fully be ruled out at this stage. Caspase-8 can also be targeted by an unrelated E3,

TRAF2, for polyubiquitylation and proteasomal degradation, in this case to shut off cell-extrinsic apoptosis (Gonzalvez *et al*, 2012).

CULLIN3-RING ligases in human disease

Given the importance of CRL3s in controlling different cellular and developmental processes, it is perhaps of little surprise that they are also linked to the pathology of various human diseases, including metabolic disorders, muscle and nerve degeneration, but also neoplastic diseases. In this regard, gene dosage alterations and expression regulation of CRL3 complex components appear to be the major underlying pathophysiological mechanisms. In addition, elaborate sequencing approaches and database-mining efforts identified a number of specific mutations in patients suffering from several diseases (Table II). This information helps to understand CRL3 pathways at the molecular level and may in the future even allow their targeting via new therapeutic approaches.

Metabolic diseases

Recent exome sequencing approaches identified numerous recessive and dominant mutations in *CUL3* and *KLHL3* genes in patients suffering from type II pseudohypoaldosteronism (PHAII) or Gordon's syndrome, a rare disease featuring hypertension due to misbalance between renal salt reabsorption and electrolyte excretion (Boyden *et al*, 2012; Louis-Dit-Picard *et al*, 2012). Previously, mutations in WNK ('with no lysine') kinases have been correlated with this pathological condition (Wilson *et al*, 2003). Interestingly, 9 of 16 dominant mutations were found to cluster within the Kelch propeller of KLHL3 and in the vicinity of the other sites implicated in direct substrate binding, suggesting that KLHL3 mutations may abrogate binding and ubiquitylation of targets normally required for modulation of renal salt $K+$ and $H+$ handling in response to physiological challenge (Boyden *et al*, 2012). This notion gains support from recent studies presenting evidence that WNK kinase isoforms may be the critical CUL3-KLHL3 ubiquitylation targets (Ohta *et al*, 2013; Shibata *et al*, 2013; Wakabayashi *et al*, 2013). Disease-causing mutations in KLHL3 abolish interactions with either CUL3 or WNK kinases, and conversely disease mutations within acidic motifs in WNK1 and WNK4 disrupt interaction with KLHL3 (Ohta *et al*, 2013; Wakabayashi *et al*, 2013). The CUL3-RhoBTB1 E3 ligase has also been implicated in hypertension and vascular smooth muscle function, via its regulation of PPARγ and RhoA/Rho-kinase pathways (Pelham *et al*, 2012), and further support for the importance of CUL3 in blood vessel homeostasis comes from the role of the CUL3–BAZF complex in regulating angiogenesis via Notch signalling (Ohnuki *et al*, 2012). Finally, CUL3-SPOP controls the stability of the pancreatic duodenal homeobox 1 (Pdx1) transcription factor, and thereby affects pancreatic β cell function in glucose homeostasis (Claiborn *et al*, 2010). Thus, the CRL3 system emerges as an important regulator of metabolic homeostasis, perhaps by regulating responses to specific stress signals.

Dystrophies

Causative mutations for autosomal dominant Retinitis Pigmentosa (adRP), a heritable form of progressive retinal dystrophy that results in blindness and visual field loss, have been identified in the *KLHL7* gene (Kigoshi *et al*, 2011). While not affecting KLHL7 dimerization, the resulting substitutions of a conserved alanine residue (A153T and A153V) in the KLHL7 BACK domain disrupt interaction with CUL3, consistent with the recently established structural requirement for the BACK domain in CUL3 complex assembly (Canning *et al*, 2013). As E3 ligase activity was strongly reduced upon mutation of this residue (Kigoshi *et al*, 2011), adaptor protein interaction with CUL3 but not adaptor protein dimerization status appears to determine CUL3 activity (see below). Similarly, Leucine 95 mutation (L95F) of KLHL9, found in patients suffering from a form of distal myopathy of skeletal muscles, results in reduced interaction with CUL3, although with less pronounced effects (Cirak *et al*, 2010). In Nemaline myopathy (NEM), one of the most common congenital myopathies, dominant mutations have been identified in the KBTBD13 protein (Sambuughin *et al*, 2010), later found to be a component of a functional CRL3 complex (Sambuughin *et al*, 2012). The substitutions R248S, K390N, and R408C are located within the β-sheets of the highly conserved second and fifth Kelch repeats and are predicted to disrupt the molecule's β-propeller structure (Sambuughin *et al*, 2010). Another BTB protein, Gigaxonin, is mutated in giant axonal neuropathy (GAN), a severe neuropathy of peripheral nerves and the central nervous system that is characterized by neurofilament accumulation and segmental distension of axons (Bomont *et al*, 2000). Here, at least one patient mutation, leading to a truncation of the Gigaxonin Kelch propeller, was demonstrated to abolish recruitment of a substrate, the microtubule-binding protein MAP1B (Ding *et al*, 2002). Further CRL3 links to neurodegeneration come from the BTB adaptor protein KCTD7 (Azizieh *et al*, 2011), which is found mutated in progressive myoclonic epilepsy (EPM3) (Van Bogaert *et al*, 2007) as well as in neuronal ceroid lipofuscinosis (NCL), a heritable lysosomal storage disease characterized by childhood-onset neurodegeneration. Here, the identified R184C variant alters KCTD7 subcellular localization patterns and abrogates interaction with CUL3 (Staropoli *et al*, 2012).

Cancer

CUL3-dependent regulation of Nrf2 pathway in cancer. As discussed earlier, Nrf2 is a master transcriptional activator of genes encoding numerous cytoprotective enzymes and is regulated by CRL3-Keap1. It has been suggested that elevated levels of cytoprotective enzymes provide tumour cells with protective capabilities against environmental stresses, a hallmark of malignancy (Gupta and Massague, 2006). Keap1 has been found to be mutated within its substrate-binding Kelch propeller domain (G364C, G430C) in a lung cancer patient and in lung carcinoma cells, respectively, reducing its affinity to Nrf2 and allowing for high constitutive activation of Nrf2 (Padmanabhan *et al*, 2006). Likewise, other lung cancer patient mutations within the Kelch domain either abolish interaction with Nrf2 (R415G) (Ohta *et al*, 2008) or inhibit Keap1-mediated degradation of Nrf2 (G333S, L413R) (Singh *et al*, 2006). Interestingly, the R272C substitution in the Keap1 BACK domain abolishes E3 activity without affecting binding to CUL3. Since R272 lies in a close proximity to those cysteines (C273 and C288) previously implicated in regulating ubiquitylation of Nrf2, this mutation might lead

to an aberrant way of binding to CUL3, and subsequent inhibition of ubiquitylation activity (Ohta *et al*, 2008). Consistent with the fact that interaction between Nrf2 and the BTB adaptor Keap1 is mandatory for Nrf2 degradation and repression, mutations have been found in lung cancer patients that specifically alter amino acids within the DLG or ETGE substrate recognition motifs of Nrf2 (Shibata *et al*, 2008). Thus, rapid degradation of Nrf2 by CUL3-Keap1 provides a molecular basis for induction of cytoprotective enzymes both in response to stress and during disease development.

Regulation of stress responses by CUL3 in cancer. Interestingly, other CLR ligases share a similar role in critical stress responses and diseases, for instance, the CUL1-dependent IκB (Amit and Ben-Neriah, 2003) and the CUL2-dependent HIF-1α (Kim and Kaelin, 2004) regulation pathways. It is intriguing that the IκB-IKKβ pathway frequently misregulated in cancer is also a target of the CRL3-Keap1 E3 (Lee *et al*, 2009; Kim *et al*, 2010), and that genetic disruptions of the CUL3 complex found in lung cancer patients, at both copy number and gene expression levels, lead to upregulation of IKKβ protein levels and activation of the NF-κB signalling pathway (Thu *et al*, 2011). Similarly, regulation of CUL3 protein levels has been linked to bladder cancer aggressiveness (Grau *et al*, 2013) and to liver tumorigenesis (Kossatz *et al*, 2010). Likewise, CUL3 impinges on hypoxia, an essential feature of the solid tumour environment. Under hypoxic conditions, HIF-1 pathways upregulate the BTB protein KLHL20, and the CUL3-KLHL20 E3-ligase targets the PML protein for degradation to potentiate HIF-1 signalling and disease progression in prostate cancer (Yuan *et al*, 2011). In this case, CRL3-dependent ubiquitylation requires prior sequential PML modification by CDK1/2 phosphorylation and Pin1-mediated prolyl-isomerization.

Biological roles of CRL3s in plants

Whether the pathways and mechanisms regulated by metazoan CRL3s are similarly under CRL3 control in plants remains unclear at present. For instance, while orchestration of the cell cycle by SCF/CRL1 complexes and the APC/C is clearly conserved in the green plant lineage (Marrocco *et al*, 2010), possible functions of plant CRL3s in these processes remain unknown. Arguing against this possibility, plants lack homologues of the BTB-Kelch proteins that regulate Aurora B in metazoans. Nevertheless, *Arabidopsis* possess Aurora kinases with localization patterns reminiscent of metazoan Aurora B and with roles in cell division plane orientation (Van Damme *et al*, 2011); and lack of CUL3 activity leads to defects in division plane orientation in the embryo (Thomann *et al*, 2005). Furthermore, RNAi depletion of MAB1, 1 of 25 MATH-BTB proteins in maize that is specifically expressed in germ lineages and the zygote, leads to chromosome segregation defects and short spindles during meiosis (Juranic *et al*, 2012). This phenotype suggests that MAB1 might function in a similar way as the katanin-degrading nematode MEL-26, although the cellular targets of this maize CRL3-MAB1 remain unknown at this stage. Irrespective of potential CLR3 functions in such basic cellular processes, plant CRL3s have been found to possess important novel functions in various plant signalling pathways and in plant hormone biology.

Regulation of plant hormone biosynthesis and signalling

Ethylene is a gaseous hormone that regulates numerous aspects of plant development, but is also important for plant responses to adverse environments, such as interactions with pathogen. ACS5, a member of the type-2 1-aminocyclo-propane-1-carboxylic acid synthases (ACSs) that catalyse a rate-limiting step in ethylene biosynthesis, was the first identified CRL3 substrate in the plant kingdom (Wang *et al*, 2004). In *Arabidopsis*, ACS5 is recognized by the Tetratrico Peptide Repeat (TPR) domain-containing BTB adaptor ETO1, leading to both its inactivation and also its degradation to subsequently repress ethylene production. Mutations of ETO1 or downregulation of CUL3 function results in both ACS5 stabilization and ethylene gas overproduction, affecting plant growth and development (Wang *et al*, 2004; Thomann *et al*, 2009). Moreover, two other Arabidopsis BTB-TPR proteins closely related to ETO1, designated as ETO1-like (EOL1) and EOL2, also negatively regulate ethylene synthesis via their ability to target ACS5 and other type-2 ACSs for degradation (Christians *et al*, 2009). The mechanisms by which ETO1/EOL1/EOL3-CRL3s recognize type-2 ACSs to decrease their stability are still not clearly defined, as initial models of a C-terminal recognition sequence in type-2 ACSs that are negatively regulated by phosphorylation, leading to stabilization of these enzymes (Chae *et al*, 2003; Wang *et al*, 2004), was subsequently called into question (Christians *et al*, 2009; Skottke *et al*, 2011).

In addition to regulating ethylene biosynthesis, CRL3s are also involved in controlling signalling by another important plant stress hormone, abscisic acid (ABA). The *Arabidopsis* genome encodes six members of the evolutionarily conserved MATH-BTB protein family (called BPM1-6) (Weber *et al*, 2005), and all six members were found to interact with a subclade of the class I homeobox-leucine zipper (HD-ZIP) transcription factors (Lechner *et al*, 2011), including ATHB6, a negative regulator of ABA responses (Himmelbach *et al*, 2002). CUL3-BPM mediates ubiquitylation and turnover of ATHB6, but this process is slowed down in the presence of ABA, affecting some of the plant responses towards ABA, in particular stomatal closure (Lechner *et al*, 2011). Note that Arabidopsis MATH-BTB proteins also interact with members of the ETHYLENE RESPONSE FACTOR (ERF)/APETALA2 (AP2) to target their proteasomal degradation, as recently described for WRINKLED1, an ERF/AP2 transcription factor involved in fatty acid metabolism (Chen *et al*, 2013).

A striking feature of the MATH-BTB class of adaptors is there selective expansion and diversification in some worm and plant species during evolution (Thomas, 2006; Gingerich *et al*, 2007). For instance, while the *Arabidopsis* genome encodes only six members of this family, there are 74 predicted MATH-BTB proteins in rice (Gingerich *et al*, 2007), and it has been speculated that this expansion may reflect positive selection in response to rapid adaption of possible targets such as pathogen proteins (Thomas, 2006; Gingerich *et al*, 2007). One major route of pathogen entry into plant cells is stomata opening, and not surprisingly pathogens evolved virulence factors to counter stomatal closure and facilitate invasion (Zeng *et al*, 2010). As noted above, *Arabidopsis* MATH-BTB mediates ABA-dependent stomatal closure and is therefore strongly expressed in guard cells (Lechner *et al*, 2011). As many microbial pathogens are

known to exploit the ubiquitin pathway to evade host defence mechanisms (Jiang and Chen, 2011), it will be of particular interest to explore possible functions of this class of CRL3 substrate adaptors in innate immunity and bacterial diseases.

Precedent for important plant immunity roles stems from another class of plant BTB proteins, which includes *Arabidopsis* NPR1, a master regulator of plant immunity that controls the onset of systemic acquired resistance (SAR) to a broad spectrum of pathogens. SAR requires the signalling molecule salicylic acid (SA), a plant hormone synthesised in response to phytopathogen challenge. Upon SA perception, NPR1 moves to the cell nucleus, where it interacts with TGA-bZIP transcription factors to induce defence gene expression. NPR1 combines a BTB domain with an ankyrin repeat motif (Cao *et al*, 1997). Interestingly, NPR1 in the nucleus is itself degraded by the proteasome in a CUL3-dependent manner, most likely to prevent activation of NPR1 target genes in unchallenged cells (Spoel *et al*, 2009). On the other hand, NPR1 is phosphorylated in SAR-induced cells, creating an IκB-like phosphodegron motif and also resulting in degradation via the CRL3 pathway to sustain maximum levels of target gene expression.

Despite its BTB domain, however, NPR1 does not directly interact with CUL3; instead, NPR3 and NPR4, two *Arabidopsis* NPR1 paralogues, are responsible for recruiting it to CUL3 (Fu *et al*, 2012). Consistently, *npr3 npr4* double mutant cells accumulate increased NPR1 protein levels and exhibit enhanced disease resistance, a phenotype opposite to the *npr1* mutation. Strikingly, NPR3 and NPR4 not only interact with CUL3 through their BTB domains, but also bind SA, thus representing the long-sought-after receptors for this plant hormone (Fu *et al*, 2012). This recognition mode resembles that of the plant hormone auxin, whose receptors belong to a six-gene clade of *Arabidopsis* FBPs including TIR1. Auxin binding to TIR1 in the context of the CRL1–TIR1 E3 complex stabilizes interactions between TIR1 and its Aux/IAA transcription repressor substrates, resulting in their ubiquitin-dependent degradation to trigger auxin transcriptional responses (reviewed in Santner and Estelle, 2009). Indeed, high levels of SA promote NPR3–NPR1 interactions, leading to NPR1 ubiquitylation and degradation at the site of infection; this prevents SAR establishment and leads to effector-triggered cell death of infected cells. However, SA has the opposite effect on NPR4–NPR1 interactions. NPR4 binds NPR1 in the absence of SA and is responsible for constitutive NPR1 degradation in non-infected cells, thus preventing spurious activation of defence gene expression. NPR4's higher affinity for SA allows it to detect lower SA levels in the cells neighbouring sites of infection, resulting in disruption of NPR4–NPR1 binding, NPR1 stabilization, and establishment of SAR (Figure 2) (Fu *et al*, 2012).

Light signalling

CRL3s have recently been implicated in phototropism, a process that allows plants to change their growth direction in response to the location of the light source. This involves a large plant-specific protein family called NRL (Pedmale *et al*, 2010), which usually contain a BTB domain, a central NPH3 domain, and a C-terminal coiled-coil domain. One of them, NPH3, is involved in early phototropism signal transduction downstream of the PHOT1/NPH1 photoreceptor, a light-activated serine/threonine protein kinase (Motchoulski and Liscum, 1999). *Arabidopsis* NPH3 interacts with CUL3 to ubiquitylate PHOT1 at the plasma membrane in response to blue light (Roberts *et al*, 2011). An interesting aspect of this process is that PHOT1 polyubiquitylation, and thus proteasomal degradation is only stimulated by high-intensity blue light, likely to allow receptor desensitization. On the other hand, low-intensity blue light promotes only PHOT1 mono- and multi-monoubiquitylation, which may trigger receptor internalization from the plasma membrane (Roberts *et al*, 2011). It will now be interesting to address the molecular basis of PHOT1 recognition with respect to light-induced structural changes, as well as the regulation of the CRL3 adaptor NPH3, whose phosphorylation status is itself light dependent (Pedmale and Liscum, 2007). Other members of the NRL family include ROOT PHOTOTROPISM2 (RPT2), which is also involved in phototropic signalling (Sakai *et al*, 2000), and NPY1/MAB4/ENP (Furutani *et al*, 2011 and references therein), which regulates *Arabidopsis* organ formation by controlling transport of the plant hormone auxin. Whether these BTB proteins also function as *bona fide* CRL3 adaptors remains to be established.

Plant BTB proteins regulate not only blue light responses, but have also been implicated in negatively regulating photomorphogenesis in response to red light (Christians *et al*, 2012). Here, a pair of so-called LIGHT-RESPONSE BTB proteins (LRB1/2) acts redundantly to limit accumulation of the red light photoreceptors phytochrome B and D, and consequently *lrb1 lrb2* double mutant plants are selectively hypersensitive to red light. Both LRB1 and LRB2 interact with CUL3A/B proteins in the nucleus, and may thus target these photoreceptors following red light-induced photoconversion and nuclear import; however, at this stage it remains to be determined whether LRB1/2 are directly involved in phytochrome turnover, for example, via light-induced direct interactions.

Mechanisms of CRL3 regulation

Ubiquitylation and fate of CRL3 substrate proteins can be controlled on various levels, such as substrate recognition, adaptor protein binding, or ligase activity. In the following, we will summarize several key mechanisms of CRL3 regulation.

Regulation by NEDD8 and CAND1

An essential feature for all CULLIN-RING ligases is the dynamic covalent modification of the Cullin scaffold by the ubiquitin-like protein Nedd8/Rub1 (Petroski and Deshaies, 2005; Merlet *et al*, 2009). Like ubiquitylation, neddylation requires the sequential action of an Nedd8-activating enzyme (NAE), the APPBP1-UBA3 heterodimer, and an NEDD8-conjugating enzyme, UBC12. In addition, DCN1 (*d*efective in *c*ullin *n*eddylation 1) cooperates with the CRL RING finger subunit RBX1 to stimulate the neddylation reaction, thus exerting an E3-like activity for NEDD8 (Scott *et al*, 2010, 2011). In a reverse reaction, NEDD8 is removed from cullins by the isopeptidase activity of the zinc-dependent metalloenzyme CSN5, a component of the eight-subunit COP9 signalosome (CSN) complex. NEDD8 attachment stimulates processes such as recruitment of ubiquitin-charged E2 enzymes and positioning of the E2

active site for ubiquitin transfer onto substrates (Duda *et al*, 2008; Saha and Deshaies, 2008). On the other hand, deneddylation promotes dissociation of CRL E3 complexes and Cullin-RING binding to the inhibitory factor CAND1 (*c*ullin-*a*ssociated and *n*eddylation-*d*isassociated 1), a regulator of CRL complex assembly that binds to un-neddylated cullins (see below) (Liu *et al*, 2002).

Several lines of evidence suggest that not just cullin neddylation, but dynamic cycles of CRL neddylation and deneddylation are key in CRL activation. CUL3 neddylation was first described in the context of CRL3-MEL26-dependent MEI-1 in *C. elegans* (Pintard *et al*, 2003a), with both neddylation and deneddylation being required for MEI-1 degradation. Likewise, while CUL3 neddylation is a prerequisite for Keap1-dependent *in vivo* ubiquitylation of Nrf2 in mammals, this process is equally affected by siRNA-mediated knockdown of CAND1 (Lo and Hannink, 2006). It is likely the CAND1 regulates CRL3s in a manner similar to its control of SCF/CRL1 complexes, where CAND1 serves as a 'protein exchange factor' that allows swapping of FBP substrate adaptors and thereby adjustment of the SCF repertoire to varying substrate demand (Pierce *et al*, 2013; Wu *et al*, 2013; Zemla *et al*, 2013). Therefore, defective Nrf2 degradation upon knockdown of the CAND1 exchange factor may reflect an inability to form additional CRL3-Keap1 modules. Quantitative proteomics results showing as much as 10% of cellular CUL3 bound to CAND1 indicate that this may indeed represent the steady-state levels of CAND1-CUL3 engagement during the course of adaptor assembly (Bennett *et al*, 2010).

CRL3 dimerization

Most (if not all) of the CRL3s dimerize through the BTB domains of their substrate recognition subunits, which can form tightly intertwined dimers with an extensive hydrophobic interface, as revealed by a crystal structure of the PLZF BTB (Ahmad *et al*, 1998; Merlet *et al*, 2009). The best-documented example of a role for such dimerization in substrate recognition comes from biochemical and structural analyses of human Keap1 (McMahon *et al*, 2006; Taguchi *et al*, 2011), where two Keap1 molecules in the homodimer bind to a single Nrf2 substrate molecule that employs two distinct binding sites (termed as DLG and ETGE motifs) with differential affinities; these dual interaction sites appear to be important to position Nrf2 in an orientation optimal for its ubiquitylation. Similar features characterize the assembly of CRL3-SPOP and CRL3-HIB complexes with their substrates, with SPOP dimerization allowing engagement of multiple SPOP binding sites in a single substrate (Zhang *et al*, 2009; Zhuang *et al*, 2009; Errington *et al*, 2012). Thus, the use of dimeric substrate recognition modules can increase the avidity for substrates with multiple degrons. Recent structural work on CUL3-KLHL11 modules illustrates how BTB dimerization occurs in the context of a CULLIN3-RING ligase complex, revealing a unique N-terminal extension in CUL3 required for interaction with the adaptor-BTB protein-specific 3-box and also providing a template for modelling quaternary CRL3 assembly such as the one that targets Nrf2 (Canning *et al*, 2013).

Dimerization of CUL3 E3s may have additional roles beyond substrate recruitment. SPOP-mediated CRL3 dimerization provides a bivalent geometry with dual docking sites for ubiquitin-charged E2 enzyme, thus enhancing both rate and processivity of polyubiquitin chain formation (Zhuang *et al*, 2009). This notion is supported by functional studies on CRL3-KLHL7, where an adRP disease mutant of KLHL7 is defective for CUL3 binding but still capable of dimerization. Via interaction with wild-type KLHL7, this mutant forms complexes containing dimeric KLHL7 but only a single CUL3 subunit, and such complexes also exhibit reduced ubiquitylation activity (Kigoshi *et al*, 2011).

In addition to dimerization, higher orders of CRL3 oligomerization have also been observed, such as formation of stable tetramers by the BTB protein KCTD11 (Correale *et al*, 2011). Moreover, recent structural studies on SPOP complexes also revealed higher-order self-assembly creating oligomeric CRL3-SPOP with enhanced E3 activity (Errington *et al*, 2012). Here again, E3 oligmerization may increase the catalytic rate of ubiquitylation by locally increasing the concentration of associated E2 enzymes. Interestingly, SPOP can also mediate formation of heterodimeric CRL3 complexes when interacting with its paralogue, SPOP-like (SPOPL). SPOPL has however restricted oligomerization capabilities, and its incorporation therefore limits higher-order SPOP-CRL3 self-assembly and attenuates E3 activity (Errington *et al*, 2012).

Subcellular sequestration of CRL3s

CRL3 substrate adaptors can become sequestered in specific subcellular compartments, separate from their substrates, to additionally control CRL3 ubiquitylation activity. As discussed earlier, the CRL3-KLHL20 substrate DAPK is stabilized upon KLHL20 sequestration in PML nuclear bodies in IFN-α-treated cells, forming the basis for IFN sensitivity of tumour cells (Lee *et al*, 2010). In a different example, CUL3 restriction to the cytoplasm stabilizes Steroid Receptor Coactivator-3 (SRC-3), a coactivator of nuclear receptors, in the nucleus under basal conditions. Retinoic acid treatment, however, leads to CUL3 translocation in the nucleus, allowing ubiquitylation and degradation of its substrate SRC-3 (Ferry *et al*, 2011).

Post-translational modification of substrates and adaptors

E3 recognition sequences in substrates, called 'degrons' (Varshavsky, 1991), frequently undergo post-translational modification that affects their recognition by substrate binding domains. This has been found particularly common for phosphorylation, which often leads to formation of a phosphorylation-dependent recognition sequence of 'phospho-degron'. For instance, signal-dependent SRC-3 ubiquitylation by CRL3-SPOP depends on a phospho-degron (Ferry *et al*, 2011; Li *et al*, 2011). Similarly, NPR1 phosphorylation in the presence of SA stimulates its CUL3-dependent turnover in *Arabidopsis* (Spoel *et al*, 2009). In other cases, however, phosphorylation inhibits rather than promotes substrates recruitment by CRL3s. For example, phosphorylation of the conserved threonine residue within the Nrf2 ETGE degron (see above) has the capability of disrupting its interaction with Keap1 and stabilising Nrf2 in cells (Lo *et al*, 2006). Similarly, substrate peptides containing phospho-serine or phospho-threonine substitutions in their SPOP binding element abolish *in vitro* binding to SPOP (Zhuang *et al*, 2009), although the occurrence and significance of phosphorylation-modulated recognition *in vivo* remains to be established.

Like target proteins, substrate adaptor proteins also are subjected to regulation by post-translational modifications. The best example is again Nrf2 regulation by CUL3-Keap1, where oxidative stress and generation of electrophiles result in modification of thiol groups in Keap1 cysteine residues, and hence attenuation of E3 activity (see Figure 2A and earlier discussion) (Cullinan *et al*, 2004; Kobayashi *et al*, 2004; Zhang *et al*, 2004); although it is still under debate how exactly Keap1 post-translational modification affects Nrf2 ubiquitylation at the molecular level (Taguchi *et al*, 2011). Finally, albeit not constituting a *bona fide* covalent post-translational modification, the earlier discussed SA binding-modulated interactions of plant BTB proteins NPR3 and NPR4 with their target NPR1 provide an additional example of CRL3 stress sensor abilities regulated at the substrate adaptor level (Fu *et al*, 2012).

Perspectives

While CRL3s are not unique in the way they are regulated or the way they target a broad range of regulatory proteins for ubiquitylation, there is still much that remains to be discovered to fully appreciate the nature of this class of E3 enzymes. One challenge is to better understand degradative versus non-degradative CRL3 roles at the mechanistic levels. While most of the BTB-containing adaptor proteins described in the current literature are linked to ubiquitin-dependent substrate proteolysis, several examples have highlighted non-proteolytic CRL3 functions. In human cells, CRL3-SPOP promotes MacroH2A mono-ubiquitylation and subsequent re-localization to the X chromosome, an important step for stable X chromosome inactivation (Hernandez-Munoz *et al*, 2005). However, as described above, the same E3 complex is also involved in ubiquitin-dependent proteolysis of other substrates like Gli2/3 (Chen *et al*, 2009a) or SRC-3 (Li *et al*, 2011), most likely via K48-linked polyubiquitin chains. Similarly, Aurora B and caspase-8 are regulated by non-proteolytic ubiquitylation and Sec31A and PLK1 are mono-ubiquitylated by CRL3s in human cells (Jin *et al*, 2009, 2012; Maerki *et al*, 2009; Beck *et al*, 2013). Another interesting example is the light intensity-dependent differential regulation of poly- versus mono-/multi-mono-ubiquitylation of PHOT1 by CRL3-NPH3 in plants (Roberts *et al*, 2011). How CRL3 E3 ligases can switch from poly-ubiquitin chain formation to mono-ubiquitylation, sometimes on the same substrate, remains to be understood. In this respect, it has to be mentioned that E2 enzymes also have major influence on the outcome of ubiquitylation and ubiquitin chain specificity. However, since the cognate E2s for CRL3s *in vivo* remain to be decisively clarified, many of the studies reported so far have relied on utilization of the promiscuous E2 enzyme UbcH5, which may have affected the respective conclusions from CRL3 *in vitro* ubiquitylation. Nevertheless, the findings on non-proteolytic ubiquitylation by CRL3s certainly expand the possible roles of CRL3s and suggest that additional cellular signalling functions of CULLIN3 ligases remain to be discovered. Future studies will also be needed to discover downstream effectors and to understand how CRL3s regulate protein localization and function in the above examples.

Another still puzzling issue concerns the real repertoire of cellular substrates for each CRL3. The degron motifs recognized by E3s are often short, poorly conserved peptide sequences within a target protein. For example, the substrate adaptor SPOP binds a five-residue peptide (called SPOP binding consensus, SBC) including a stretch of serine/threonine residues, which is present in several of its substrates, such as Puc, MacroH2A, Ci, and Daxx (Zhuang *et al*, 2009). However, these sequences are not sufficiently informative to easily identify them at the proteome level, rendering bioinformatics-based CRL3 substrate prediction uncertain, if not outright impossible. Therefore, various strategies to establish novel high-throughput approaches for identifying E3 substrates have been proposed (Yen and Elledge, 2008; Merbl and Kirschner, 2009). Given that substrate phosphorylation may not be a general prerequisite for CRL3 substrate recognition, *in vitro* ubiquitylation performed with commercially spotted protein arrays may offer a convenient approach for such studies in this case, as illustrated by the successful identification of several potential CUL3-KLHL22 substrates, many of which are involved in cell-cycle functions (Beck *et al*, 2013).

Finally, an intriguing question concerns the structural complexity of CRL3s. As indicated above, a hallmark of CRL3s is their capacity to dimerize through their substrate receptors. In particular, homodimerization is well established for a number of these complexes, but heterodimerization through related MATH-BTB subunits has also been reported (Errington *et al*, 2012). Moreover, we have recently obtained evidence for *in vivo* heterodimerization among all six MATH-BTB proteins in *Arabidopsis* (our unpublished results). Therefore, one may speculate about the actual heterodimerization potential within larger, expanded classes of CRL3 substrate adaptors in some metazoan and plant genomes, such as the predicted 52 human BTB-Kelch proteins, the 74 members of the rice MATH-BTB family, or the ~30 NPH3-BTB proteins in *Arabidopsis* (Thomas, 2006; Gingerich *et al*, 2007; Canning *et al*, 2013). Furthermore, some substrates themselves are also known to dimerize, such as dimerization of the transcription factor AtHB6 with its paralogues AtHB5 and AtHB16 (Lechner *et al*, 2011). It is therefore tempting to speculate that through combinatorial

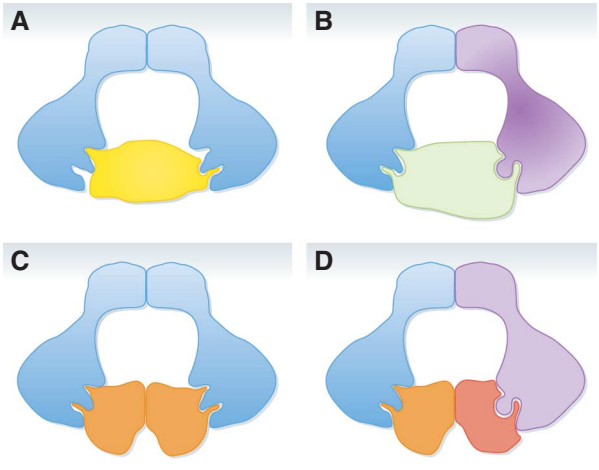

Figure 3 Speculative models on substrate accommodation by BTB protein dimerization. The model in (**A**) corresponds to the well-described binding of the Nrf2 transcription factor via two different binding sites to the Kelch domains of the Keap1 homodimer (McMahon *et al*, 2006). Models (**B**–**D**) represent speculative variations involving substrate recognition by BTB protein heterodimers (**B**) or cases considering both BTB protein and substrate homo- and hetero-dimerization (**C**, **D**).

heterodimerization at the levels of both adaptors and their substrates, CRL3s may be able to dramatically diversify their substrate repertoire and to adapt their recognition potential in many ways (Figure 3).

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Conflict of interest

The authors declare that they have no conflict of interest.

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