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Regulation of NF-κB by ubiquitination and degradation of the IκBs

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Summary: The nuclear factor-κB (NF-κB) signaling pathway is a busy ground for the action of the ubiquitin–proteasome system; many of the signaling steps are coordinated by protein ubiquitination. The end point of this pathway is to induce transcription, and to this end, there is a need to overcome a major obstacle, a set of inhibitors (IκBs) that bind NF-κB and prohibit either the nuclear entry or the DNA binding of the transcription factor. Two major signaling steps are required for the elimination of the inhibitors: activation of the IκB kinase (IKK) and degradation of the phosphorylated inhibitors. IKK activation and IκB degradation involve different ubiquitination modes; the latter is mediated by a specific E3 ubiquitin ligase $SCF^{\beta\text{-}TrCP}$. The F-box component of this E3, β-TrCP, recognizes the IκB degron formed following phosphorylation by IKK and thus couples IκB phosphorylation to ubiquitination. $SCF^{\beta\text{-}TrCP}$-mediated IκB ubiquitination and degradation is a very efficient process, often resulting in complete degradation of the key inhibitor IκBα within a few minutes of cell stimulation. *In vivo* ablation of β-TrCP results in accumulation of all the IκBs and complete NF-κB inhibition. As many details of IκB-β-TrCP interaction have been worked out, the development of β-TrCP inhibitors might be a feasible therapeutic approach for NF-κB-associated human disease. However, we may still need to advance our understanding of the mechanism of IκB degradation as well as of the diverse functions of β-TrCP *in vivo*.

Keywords: *IκB degradation, NF-κB activation, β-TrCP, ubiquitin, transcription regulation*

Introduction: IκB degradation as a key regulatory mechanism in NF-κB activation

Following the discovery of nuclear factor-κB (NF-κB) by Sen *et al.* in the Baltimore laboratory (1, 2), the next remarkable milestone in NF-κB research was demonstration of the NF-κB cytoplasmic inhibitor IκB (Inhibitor of NF-κB) (1–3). Using simple detergents, Baeuerle was able to dissociate the hypothetical inhibitor from the latent transcription factor, thus liberating the DNA binding activity of NF-κB (3). This immediately increased interest in identifying the inhibitor and elucidating a physiologic mechanism for liberation of NF-κB from the inhibitory effects of IκB (4). Subsequent experiments in cell lines suggested that stimulus-induced inhibitor of NF-κB (IκB) phosphorylation triggered release of an associated

NF-κB, which could account for physiological activation of the transcription factor (5). Yet, later experiments indicated that IκB phosphorylation was insufficient for NF-κB activation (6–9), and IκB degradation preceded NF-κB activation (10–13). However, it was Henkel *et al.* (14) who proved that IκB degradation is necessary for NF-κB activation; blocking IκB degradation prevented TNF-induced NF-κB activation. Elucidating the mechanism of IκB proteolysis followed, first through studies of Maniatis and Goldberg (15), who showed that proteasome inhibitor treatment abolished IκB degradation. Soon after, the Maniatis, Ben-Neriah, and Ciechanover laboratories (8, 15, 16) showed that signal-induced ubiquitination and proteasomal degradation of IκB was required for NF-κB activation. These findings implicated for the first time ubiquitin-dependent proteolysis as an integral step of signal-induced transcriptional activation, yet the basis for specifically targeting IκB, a single component of protein complex for degradation, while sparing the rest of the complex remained to be shown. Sequence homology comparisons in conjunction with site-directed mutagenesis revealed a high degree of conservation in a short six amino acid N-terminal sequence of the IκBs. Among these, two serine residues were consistently phosphorylated after phorbol ester stimulation, and their mutagenesis abolished signal-induced IκBα ubiquitination and proteasomal degradation (8, 17). This information geared the subsequent molecular characterization of both the IκB kinase and ubiquitin ligase (E3).

Personal and historical narrative

I (Y.B-N) had the privilege of attending the birthday of NF-κB in the Baltimore laboratory, when Ranjan Sen identified NF-κB as a lymphoid-cell DNA binding factor and then documented its signal-based induction in different cell types. Several colleagues of mine at the laboratory immediately realized the importance of the discovery, and eventually redirected their research efforts to study NF-κB. Gary Nabel implicated the factor in the transcription of human immunodeficiency virus (HIV) RNA, and Mike Lenardo documented the integration of NF-κB in enhancer functions of inducible genes. It started to be crowded around NF-κB in the Baltimore laboratory, and I thought I had been lucky resisting the temptation to join in. I was busy working on an exciting project, cloning of the proto-oncogene c-Abl and characterization of the leukemia oncogene Bcr-Abl, and followed the NF-κB excitement as an outsider. It took another 10 years to convince myself to join the NF-κB club through a fairly convoluted route.

An important milestone in NF-κB research came about after Partick Baeuerle joined the Baltimore laboratory. His work yielded the basic principle of NF-κB activation: dissociation of an inhibitor as a trigger for converting the latent transcription factor to an active one. Whereas this principle was initially documented nonphysiologically, first using detergents by Baeuerle and then protein kinase C (PKC) phosphorylation by Sankar Ghosh, it took a few more years to understand the physiologic mechanism of activation, at which phase I finally got trapped in the NF-κB net. It all started with a bizarre tyrosine kinase LTK. At the big rush for identifying new kinases in the mid-1980s, I thought it would be useful having my own baby kinase, as a starting project for the new laboratory I opened in Jerusalem after my postdoctoral term. I took advantage of a successful phage library I previously prepared in the Baltimore laboratory and cloned what I thought was a mouse homologue of the chicken ROS tyrosine kinase receptor. I called it LTK, a reminder of its origin, a mouse lymphocyte cell line 70Z/3, and found that a major expressed form of the gene in 70Z/3 cells was composed of a kinase domain, a transmembrane region, and a very short extracellular domain. When Asne Bauskin, my first PhD student expressed it, first in human COS/7 and then in other cell lines, we noticed that it never emerged out of the endoplasmic reticulum (ER), a good excuse for missing an extracellular ligand-binding domain. How do you then activate a membrane-inserted tyrosine kinase devoid of a ligand-binding domain? We tried various cellular maneuvers and found a trick—oxidative stress! The ER internal environment is very responsive to redox changes, and luckily, inducing an oxidative environment led to disulfide bond rearrangement, dimerization, and activation of LTK.

At the time we characterized the redox-responsive LTK, Patrick Baeuerle was very interested in oxidative stress responses and thought that LTK might be the link connecting oxidative cellular changes to NF-κB activation. Thomas Henkel, Patrick's PhD student, came to visit our laboratory for testing this hypothesis. The readout could have been very simple, observing tyrosine phosphorylation of IκB under oxidative damage in conjunction with LTK activation. Irit Alkalay, my PhD student, and Thomas invested a great effort in analyzing Western blots of oxidized cells, but to our great disappointment, even the tumor necrosis factor (TNF)-stimulated controls did not work out; the IκB signal disappeared whenever stimulated cells were assayed. We only realized what had happened after Thomas went back to his laboratory and there found the means of preserving the elusive IκB signal, i.e. treating TNF-stimulated cells with the inhibitors TPCK and PDTC

(likely IKK inhibitors, then considered proteolysis blockers). This was a truly exciting discovery; it had nothing to do with LTK or with oxidative stress, but matched well other findings of Irit, who parallel to other investigators, also found that phosphorylation of the NF-κB/IκB complex was not sufficient to release the inhibitor. Something else was required, i.e. IκB degradation (6–8, 18). Blocking IκB degradation with TPCK and PDTC abolished NF-κB activation.

Shortly after this key observation was made, I discussed it with a friend, Aaron Ciechanover, who, together with Avram Hershko, discovered the ubiquitin system. Naturally, Aaron suggested looking at ubiquitination, as a possible mediator of proteasomal degradation. A strong support for Aaron's idea came from Tom Maniatis' laboratory, showing that proteasome inhibitors blocked TNF-stimulated degradation of IκB. In 1995, several groups showed that phosphorylation at IκBα serine residues 32 and 36 was necessary for the inducible degradation of IκBα (17, 19–21). However, the mechanism coupling IκB phosphorylation to ubiquitination had not been resolved then. Using a cell-free IκBα degradation assay, Irit Alkalay and Amir Orian, a student of Aaron, proved that ubiquitination was necessary for the degradation of phosphorylated IκBα (16). A similar observation was made then by the Maniatis laboratory (8). At that point, I realized that we might have embarked onto an intriguing interface, a perfect match between protein phosphorylation, my previous field of interest, and ubiquitination, a relatively unexplored signaling theme at that time. Around the same period, I spent a sabbatical at Michael Karin's laboratory in San Diego and met Frank Mercurio, a former student of Karin and a researcher in the company Signal Pharmaceuticals. Following discussions with Frank and Michael, we convinced Signal Pharmaceuticals to invest an effort in NF-κB research. Frank engaged himself in looking for the IκB kinase (IKK) with Michael and at the same time helped us in identifying the IκB ubiquitination components. 3–4 years later, these collaborations yielded both the IKK (22, 23) and β-TrCP, the IκB ligase (24).

In the early to mid-1990s, ubiquitin and signaling were mostly distinct entities. Varshavsky *et al.* (25) had already identified the N-end rule, a recognition sequence for the ubiquitin-proteasome system and later termed it a degron (26). Kirschner (27) had subsequently identified the cyclin destruction box yet signaling-inducible degrons were unknown. Listening to Tom Maniatis at a Cold Spring Harbor meeting, I learned about the sequence conservation at the N-terminal region of the IκB proteins, a feature of the known degron motifs, and I then thought we should study the putative IκB degron in detail. The task was assigned to my student Avraham (Bami) Yaron, who together with Irit Alkalay and Ada Hatzubai skillfully set an *in vitro* ubiquitination assay of IκBα, where ubiquitin conjugation was dependent on prior TNF-stimulated phosphorylation of the NF-κB inhibitor. Ada contributed her magic fingers to that task, producing some of the most efficient *in vitro* ubiquitination reactions ever seen with full conversion of IκBα to polyubiquitinated species. Bami acquired a set of phosphorylated IκBα N-terminal peptides from Frank Mercurio and proved that even a short 7-mer phosphopeptide encompassing the putative degron was sufficient to compete with signal-phosphorylated full-length IκBα and to block the *in vitro* ubiquitination of the intact protein. More impressively, microinjecting these phosphopeptides to TNF-stimulated cell lines blocked NF-κB translocation into the nucleus and target gene expression (28). We thus confirmed the idea of inducible, protein modification-dependent degrons, as of today, a known prevailing signaling step in many cellular pathways. However, the major task was still ahead of us, identifying the ubiquitin system component that mechanistically couples phosphorylation to ubiquitination. Hershko and Ciechanover had already discovered in the early 1980s that the UPS (ubiquitin proteasome system) component that endows the specificity to the protein degradation process is commonly the E3 ubiquitin ligase (29). We therefore presumed that we ought to look for a specific E3, yet shortly realized that we were not alone at this front. Many laboratories rushed then to try and isolate the IκBα-E3 ligase.

Whereas we learned a lot from the Ciechanover group, our biochemical skills proved insufficient for successfully running a traditional purification scheme for identifying the putative E3. All we found using biochemical fractionation was UBC5, a ubiquitin-conjugating enzyme (E2) of IκBα, which did not solve the question of ubiquitination specificity but later proved valuable for confirming the E3 identity. As an alternative to exhaustive purifications, we finally thought of using our winning trick, taking advantage of the IκBα ubiquitination-inhibitory phosphopeptides. Bami and Ada precipitated the IκBα-NF-κB complex from HeLa cell lysates, phosphorylated it *in vitro* with a recombinant active IKK provided by Mercurio, and used the phosphorylated complex as bait for fishing the elusive IκBα-E3. The degron peptides were then used to specifically release the E3 from the bait. However, fishing is one business and recognizing the trapped fish requires different skills, the latter provided by Jens Andersen from Matthias Mann's lab. Matthias has been a pioneer in protein mass spectrometry (MS) analysis, and luckily, we met him through Mercurio. Comparative MS analysis is a common practice today, but in 1998, locating a protein band of interest

on a gel for MS analysis was rather challenging. Even upon peptide elution, there were still many eluted contaminants and leaking NF-κB components. Jens had to search thoroughly among the bands cut by Bami for a peptide-ion that was specifically eluted by the right IκBα peptide. Despite all these hurdles, we succeeded and identified a few peptide tags derived from the Slimb E3 homolog, β-TrCP, a human protein, which shortly before had been identified as a binding protein of the phosphorylated HIV-Vpu1 protein (*Fig. 1*). Vpu1 shared with IκBα the degron motif, through which it targets a Vpu1-associated cellular protein CD4 for proteasomal degradation (30). Once we realized that we fished β-TrCP, we knew it was the long-sought IκB-E3 and could then easily validate its role in IκB degradation and NF-κB activation by various transfection-based assays.

Shortly after publishing the identification of β-TrCP as the specificity component of the IκB-E3 (24), we learned how intense the race for the E3 was. Other interested laboratories skipped any purification. Based on Jiang and Struhl's discovery of *Drosophila* Slimb (31) and its putative role in β-catenin degradation (32), they guessed the E3 identity. Numerous publications then followed, showing that β-TrCP has many substrates, from hormone receptors to cell cycle regulators (33–35). Our group turned its attention to one particular substrate besides IκB, β-catenin, and elucidated the nature of the destruction complex targeting β-catenin for degradation in the Wnt signaling pathway. My student Sharon Amit together with Irit Alkalay (36) identified casein kinase 1 (CKI) as a destruction complex component that initiates the phosphorylation-cascade necessary to create the β-catenin degron. Recently, Ela Elyada and Ariel Pribluda (37) documented the necessity of CKIα phosphorylation for β-catenin degradation in a mouse model of intestinal cancer. Naama Kanarek proved that β-TrCP is essential for NF-κB activation *in vivo*, as well as for the integrity of many tissues; ablation of the two β-TrCP isoforms in the mouse is lethal even upon tissue-specific targeting (N. Kanarek, unpublished data). Surprisingly, very little β-TrCP is required to maintain its tissue specific functions. Using a combined targeting method based on pan-tissue knockout of β-TrCP1 and partial RNA interference (RNAi)-mediated knockdown of β-TrCP2, Naama in collaboration with Michele Pagano showed that most mouse tissues do well on minute residual levels β-TrCP2, with the exception of the testis. Testicular differentiation was completely blocked in the doubly targeted mice yet surprisingly was relieved upon depletion of a single β-TrCP substrate, Snail 1, which accumulation proved detrimental and solely responsible for the testicular phenotype (38).

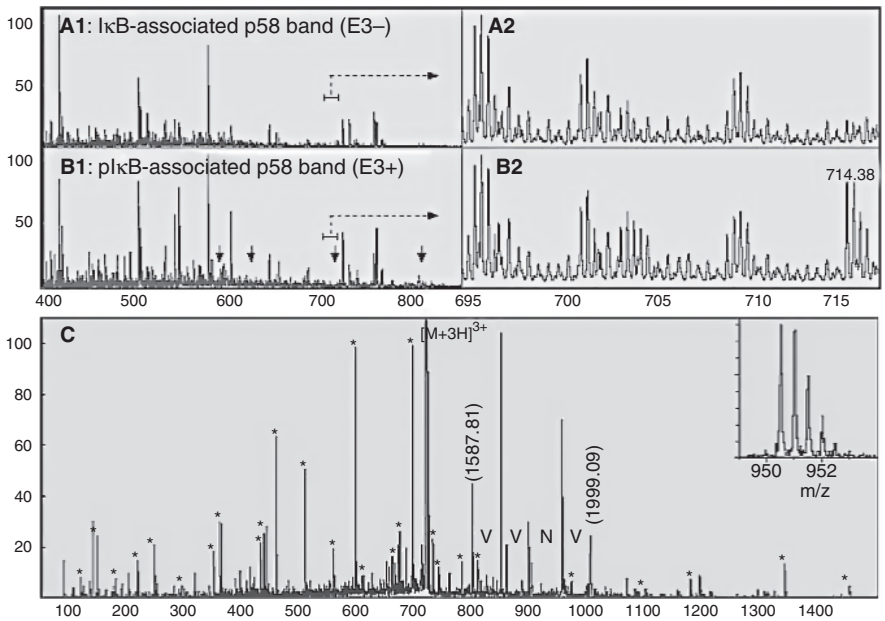

***Fig. 1.* Identification of β-TrCP by comparative mass spectrometry (MS) analysis of IKK-phosphorylated- vs. non-phosphorylated IκB-bound proteins (24).** A1, B1, nanoelectrospray mass spectrum of the unseparated tryptic peptide mixture from the 58K gel band derived either from peptide elution of non-phosphorylated (A1), or IKK-phosphorylated IκB complex (B1). Peaks marked by arrows were fragmented and eventually identified as peptides originated from β-TrCP. The bar indicates the region enlarged in A2 and B2. The peptide at mass-to-charge (m/z) 714.38, which was only present in the phospho-IκB fraction, was selected for MS-MS sequencing. C. Fragmentation spectrum of the distinctive peptide identified in B2. A sequence tag was assembled from a series of doubly charged fragment ions and searched in the NRDB protein database for a matching pattern. Fragment masses calculated for the retrieved β-TrCP sequence AAVNVVDFDDKYIVSASGDR are indicated.

While studying β-TrCP *in vivo*, we also turned our attention to exploring the role of NF-κB in mouse models of human pathologies, mostly through inducible expression of a non-degradable IκB, the so-called 'super-repressor'. We learned that NF-κB has a far wider tissue function than previously appreciated, with a capacity to orchestrate an innate immune response from an epithelial stage. Iris Lavon (39) showed that *Listeria* infection could be deleterious upon blocking NF-κB activation in liver parenchymal cells. This is hardly surprising today but was quite provocative at the end of the previous century, when the prevailing thought was that NF-κB exercises its immunity function solely through immune and inflammatory cells. Later experiments of Eli Pikarsky (40) using the super repressor in a mouse model for hepatitis-associated hepatocellular carcinoma demonstrated one of the first molecular links between inflammation and cancer, showing that NF-κB functions as a tumor promoter in cancer. Since then, we have learned that animal models could many times provide a much more comprehensive view of the physiology and pathology of signaling pathway actions than tissue culture experiments. This is mainly due to heterotypic cell interactions, such as between epithelial and stromal/inflammatory cells, which are difficult to analyze in systems other than animal models. Hence, we have gone a long way from purifying β-TrCP to recent studies of this E3 and NF-κB in animal models of human disease but have enjoyed it throughout.

The IκB E3 ubiquitin ligase

IκBα is a relatively stable protein in resting cells with a half-life of approximately two and a half hours. Following TNF stimulation however, it only lives for 1.5 min (14). The signal that transforms IκBα to a short-lived protein in stimulated cells is the phosphorylation of serine residues 32 and 36 (8, 28). Using a set of specific phosphopeptides, Yaron *et al.* (28) showed that the phosphorylation-based motif (DpSGXXpS) of the IκBs is sufficient for targeting IκB to ubiquitination: doubly phosphorylated peptides encompassing the motif effectively blocked the ubiquitination of IκB *in vitro* and their microinjection to TNF-stimulated cells eliminated the translocation of NF-κB into the nucleus and target gene expression. These experiments set the stage for elucidating the basis for recognition of phosphorylated IκB and identifying the components of the IκB destruction complex.

Protein modification can create a recognition motif for the UPS, termed the degron, recognition of which relies on the ubiquitin system component E3, also known as ubiquitin ligase. E3s have the capacity to both recognize a degron and catalyze, either by themselves or together with E2s, ubiquitin conjugation to the substrate carrying the degron (41). Rapid degradation of phosphorylated IκBα requires an E3 with features accounting for distinguishing the inducible degron from the non-modified sequence motif and guiding the rapid ubiquitin conjugation of the substrate. An SCF-type E3 possesses these required characteristics. SCF E3s belong to the larger CRL (Cullin-RING ligases) E3 family. CRLs are a large family of multi-component E3s consisting of a core cullin protein bound to a RING finger protein (Rbx1/2), and an interchangeable substrate-binding adapter protein. There are 7 cullins and ~600 adapters in the human genome. SCF E3s in particular engage phosphorylated proteins and thus distinguish phosphorylated from nonphosphorylated substrates. For that property, they must have enough versatility to cope with many different inducible degrons and a capacity to efficiently guide an appropriate E2 for ubiquitin conjugation of the substrate. This is achieved by the assembly of the different SCF components, where the Skp1 and Cul1 (S and C of SCF) serve as scaffolds, the variable F-box protein (F in SCF) is the recognition subunit, and the Ring protein Roc1/Rbx1 is both an adapter for an associated E2 and component of the catalytic conjugation machinery (*Fig.* 2). This complex structure provides both the specificity and the high degree of processivity to the ubiquitin conjugation reaction. High processivity, based on an efficient dynamic association of the E3 with the E2 provides both the speed and the robustness of the conjugation reaction, needed for maximal ubiquitination and degradation of a substrate (42, 43).

Substrate recognition by CRLs and SCFs is predominantly the function of the F-box proteins (FBPs). There are approximately 100 FBP genes in a mammalian genome encoding proteins that share a conserved 40-amino-acid domain called the F-box domain, which connects to the rest of the SCF complex through Skp1 (44). The substrate-binding domain of the FBP is commonly positioned directly carboxy-terminal to the F-box domain in the sequence, and no FBP has more than one F-box domain (45). FBPs are classified according to their substrate-binding motif. The FBW family has a WD-40 repeats domain, structured as β-propeller, which recognizes motifs containing phosphorylated serines or threonines (42). The FBL family has a leucine-rich repeat in an arc-shaped α-β-repeat structure. A non-obligatory preference of substrate recognition by FBL is the pre-phosphorylation of the substrate or, at times, its attachment to other proteins in a complex (46). Other FBPs are termed FBX and contain a variety of protein–protein interaction domains. Whereas SCF proteins primarily target phosphorylated proteins, other CRLs have the

capacity to recognize substrates following other post-translational modifications, including glycosylation and hydroxylation (47–49). This mode of regulation enables individual recognition of many substrates by the same FBP over different cellular conditions.

Taking into account these FBP features, it is no wonder that the specificity component of the IκB-E3 is the F-box protein β-TrCP. It is a homolog of the *Drosophila* protein Slimb, which, had been identified in a genetic screen as a likely E3 for β-catenin and *Cubitus Interruptus* (Ci) (31). Human β-TrCP was initially identified as a binding partner of Vpu1, a small HIV protein sharing the IκB degron, which is created following CKII phosphorylation and assists in the degradation of another Vpu1-associated protein, CD4 (30). Taking advantage of IκB-degron-based phosphopeptide competition assay, Yaron *et al.* (24) identified β-TrCP by mass spectroscopy as a protein that specifically interacts with the IκB degron (*Fig.* 1). Several other laboratories (50–52) then demonstrated that β-TrCP is the substrate binding (receptor) subunit of an SCF-type E3 ligase (42) (*Fig.* 2). β-TrCP was subsequently shown to control the proteasome-mediated degradation of other IκBs, IκBβ and IκBε (53, 54), and of NF-κB1 p105 (55–57), as well as the signal-induced processing of NF-κB2 p100 to p52 by the proteasome (58).

Substrate recognition by β-TrCP

β-TrCP, one of the best-characterized FBPs, is mainly located in the nucleus (59) but likely also functions in the cytoplasm (31). It binds a diverse list of phosphorylated substrates, including regulators of inflammation and cell fate (the IκBs), development and tissue organization (β-catenin, Snail), many cell cycle regulators (Emi1, Wee1), and DNA damage responders (Claspin, Cdc25A) (31, 60–64). In spite of β-TrCP's pivotal contribution to many oncogenic cellular pathways, there are only a few known cases of mutations in the β-TrCP genes in human malignancies; one example is some cases of multiple myeloma (65). This might reflect the essential physiological role of this E3 or the redundancy of the two β-TrCP paralogs encoded by two different genes. The two paralogs are thought to be identical in their known biochemical features, considering their high degree of sequence homology (77%) and virtually identical substrate recognition 'pocket' (66). The redundancy may pertain to the mild phenotype observed in the *in vivo* ablation of β-TrCP1; the two best-characterized substrates of β-TrCP, β-catenin and IκB, do not accumulate in β-TrCP1-deficient mouse embryonic fibroblasts ($MEF^{\beta\text{-}TrCP1-/-}$) (61).

Rarely there is a detailed mechanistic understanding of the E3-substrate interaction as with β-TrCP. Peptide inhibition studies clearly defined the degron sequence based on dual phosphorylation, for ubiquitination of phosphorylated IκBs. These studies also specified phosphorylation-based rather than charge-based interactions, as substituting phospho-serines of the degron with aspartic or glutamic acid abolished IκB recognition (28). These degron characteristics were nicely confirmed later by the crystallography analysis of Pavletich and colleagues (67), who also explained how the degron fits into the substrate-binding pocket of β-TrCP. This has been achieved by the co-crystallography of β-TrCP with a phosphorylated β-catenin peptide, sharing with IκB the core degron motif DpSGΦXpS (GΦX referred to as a 'spacer', **Φ** stands for a hydrophobic, and X is any residue). Several interesting binding parameters are revealed through the structure: phosphopeptide binding is mediated by one face of the β-propeller, a structure created by the seven WD repeat domain of β-TrCP, which is sufficient for maintaining interaction with the different substrates. Other F-box protein domains in the WD vicinity (e.g. the F-box, the F-box-WD linker and the dimerization domain) or other SCF components do not participate in substrate binding. A central groove running through the middle of the WD propeller structure accommodates the degron. All six degron residues establish contacts with β-TrCP; the side chains of the aspartic acid, the hydrophobic residue (**Φ**) the backbone of the glycine, and the spacer residues (X) insert the farthest into the groove, making intermolecular contacts in a mostly buried environment. The phosphate groups of the two serines bind at the rim of the groove and together with the aspartic acid make the largest number of contacts with β-TrCP residues surrounding the groove via hydrogen bonds and electrostatic interactions. All seven WD repeats of β-TrCP contribute contacts to the bound peptide. Pavletich's data explain well the interaction of β-TrCP with IκBs and other canonical degron motif substrates, yet a remaining interesting question is how the E3 can accommodate peptides containing atypical degrons, such as p105, which contains a longer spacer. A hint to that end can be found through Rosetta software analysis (68). A comparison of the structural models of phosphopeptides derived from β-catenin suggested that peptides of different spacer length could be accommodated into the groove. A hydrophobic residue (isoleucine, leucine, valine, phenylalanine), which follows the glycine in all IκB-like degron sequences is packed against a hydrophobic patch in the groove. The longer the binding motifs, the deeper into the groove this hydrophobic residue will be inserted. This docking information not only

may explain substrate specificity but might also be useful in designing β-TrCP blocking agents as NF-κB inhibitors (see below).

Regulation of β-TrCP function

The regulation of β-TrCP activity is primarily oriented to generating its degrons. This feature is provided by the signal dependent phosphorylation of nearly all known β-TrCP substrates (34, 42, 69). Having many unrelated cellular substrates, β-TrCP has to adapt itself to the turnover needs of many proteins in response to different stimuli, sometimes all at once. Different substrates demand the attention of β-TrCP at different or similar temporal, spatial, and physiological contexts. Some substrates, such as the IκBs, must be degraded quickly following stimuli, while others, like β-catenin, are degraded continuously and stabilized upon stimulation. β-catenin and the IκBs may therefore be ubiquitinated distinctly but sometimes simultaneously, necessitating that β-TrCP would be available at all times. Conceivably, under certain physiological changes, the demand for the E3 may exceed its preexisting cellular levels requiring a boost to its expression. There is however only limited data concerning the transcriptional regulation of β-TrCP. One example is the regulation of β-TrCP by the Wnt pathway at the mRNA level, both by modulation of the rate of transcription and the stabilization of mRNA (70, 71). β-TrCP1 mRNA stability is also increased following c-Jun N-terminal kinase (Jnk) activation in a transcription-independent manner (72). The transcriptional regulation of β-TrCP2, however, is probably different, with an inhibitory Wnt stimulus and a stimulatory mitogen-activated signaling resulting in a β-TrCP2 transcriptional decrease (72). However, the physiological relevance of these control modes has not been established. Furthermore, unless having different substrate specificity, opposing transcriptional regulation of the two paralogs may offset each other.

Since the entire SCF complex acts as a single functional E3 unit, it is difficult to predict the outcome of a change in the levels of a single subunit with respect to the E3 function. For example, comparable tumorigenic phenotypes were obtained for two transgenic mice, one overexpressing the WT β-TrCP1 and the other expressing a dominant negative mutant of the same molecule, exemplifying the problems inherent in upregulation of a single β-TrCP subunit in isolation (73). Whereas a dominant negative mutant lacking the F-box would fail to assemble an SCF complex and thus would sequester phosphorylated β-TrCP substrates, for an overexpressed WT β-TrCP, the stoichiometry of the other components in the SCF complex might be inadequate, resulting in the apparent dominant-negative function of WT β-TrCP transgene. The need for balanced expression of the SCF subunits thus questions the significance of modulating β-TrCP expression levels as a major mode of regulation of the E3 activity.

As a part of a Cullin-Ring ligase (CRL)/SCF complex, β-TrCP activity is also subject to regulation directed at other components of the complex. The Nedd8 ubiquitin-like molecule regulates the assembly and catalytic activity of the SCF complex (74–76). Nedd8 attachment stimulates multiple CRL ubiquitin E3 activities, including binding to E2-ubiquitin, enhancing ubiquitin transfer from the E2 active site, and positioning the E2 active site adjacent to the substrate (77). One mechanism by which Nedd8 modification of Cul1 stabilizes the SCF complex is by preventing the binding of Cand-1. Cand-1 binds to cullin-Rbx1 complex and occludes Skp1, thereby preventing SCF assembly (78). Cleavage of Nedd8 by the COP9/signalosome (CSN) will allow the binding of Cand-1 and decrease the SCF activity (79). Another Neddylation-based mechanism is through reorienting the Ring-E2 position for better ubiquitin transfer to the substrate (80). Thus, both the rate of assembly and the conformation of an SCF complex are subject for regulation, with the purpose of preventing spurious activity of the E3 complex on the one hand and enabling its rapid activation on demand. Indeed, studies with a small molecule neddylation inhibitor in cell lines and mouse xenografts attest to the importance of this modification to CRL function (81)

Another level of SCF regulation that has been proposed is based on the interaction of β-TrCP with the abundant nuclear protein hnRNP-U. This is reminiscent of a pseudo-substrate control, as hnRNP-U binds to the E3 but is not ubiquitinated (59). hnRNP-U-association may control β-TrCP both by maintaining it in the nucleus and by raising its substrate-binding threshold. The latter may limit degradation of low affinity substrates, such as nonphosphorylated or partially phosphorylated substrates. Examples of a low affinity substrate are IκBα molecules that are phosphorylated only at a single serine of the β-TrCP degron (Ser36 by IKKε, and Ser32 by Rsk1) (82–84) or β-catenin tumor mutants at a degron residue (85). Whereas these molecules could have been targeted by β-TrCP although at low affinity, they would fail to compete effectively with saturating levels of hnRNP-U and will therefore escape β-TrCP ubiquitination. A similar mode of regulation was proposed for the E3 activity of the anaphase-promoting complex/cyclosome (APC/C), where Emi1 plays the pseudo-substrate role (86).

One missing piece in the NF-κB activation puzzle is the site of IκB ubiquitination and degradation. It is underscored by

the discordant subcellular localization of the IκBs, both the typical IκBs and the NF-κB precursors that are mostly cytoplasmic, while the E3 β-TrCP is mostly nuclear. A major fraction of β-TrCP is sequestered in the nucleus by the pseudosubstrate hnRNP-U and coimmunoprecipitation studies show that the hnRNP-U-β-TrCP complex is poised for IκBα ubiquitination upon exchange of the low affinity hnRNP-U pseudosubstrate with the high affinity phosphorylated IκBα (59). SCF components other than β-TrCP, as well as the proteasome, have been reported to function in the nucleus (34, 87), targeting various substrates for ubiquitination and degradation, including the p53 inhibitor Mdm2, the CDK inhibitor Far1, and others (87). How, then, would a nuclear E3 target cytoplasmic substrates? One possibility is that the location of the components is not fixed, and either the substrate or the E3 may switch position between the nucleus and cytoplasm, randomly or on demand. Indeed, IκBα-NF-κB complexes shuttle continuously between the cytoplasm and the nucleus of unstimulated cells (88–91). This shuttling is mediated by a nuclear export signal (NES), primarily that on the N-terminus of IκBα and a nuclear localization signal (NLS) of p50 and p65 (92). Whereas it has been reported that IκBβ, which lacks such NES, is capable of masking the p50/p65 NLS (92) and does not shuttle similarly to IκBα (93), IκBβ-NF-κB complexes are also detected in the nucleus, even bound to chromatin (94). As for IκBα, its shuttling is apparently unperturbed as long as IKK is not activated. Once IκBα is phosphorylated via activated IKK, it is recognizable by β-TrCP, following which IκBα ubiquitination and degradation releases NF-κB. Whereas the phosphorylation of IκBα is presumably a cytoplasmic event (69), the subcellular site of its ubiquitination and degradation is unknown. Notably, the physiological relevance of the shuttling mechanism has mainly been supported by experiments based on leptomycin B treatment, an inhibitor of IκBα nuclear export (89), yet other influences of this drug have not been excluded. Any activation model entailing steady state shuttling must therefore be taken with a grain of salt.

Degradation of the different IκBs

All IκBs (IκBα, IκBβ, and IκBε, and the ankyrin-containing Rel genes) are targeted for ubiquitination by β-TrCP, but following cell stimulation, they are degraded with different kinetics (4). IκBα is the fastest to degrade [less than 5 min in TNF-stimulated HeLa and 15 min in lipopolysaccharide (LPS)-treated Jurkat cells] and IκBε the slowest (60-90 min in phorbol myristate acetate (PMA)/ionomycin or LPS-stimulated Jurkat cells)(95). It has been difficult to define the major factor that influences these kinetics, and the limiting factor in different physiological contexts might vary. One major factor is likely the stimulus itself, but even when examining one of the most effective stimuli, TNF, there is little correlation between the applied dose and the kinetics of IKK phosphorylation activity or IκB degradation (96). IKK itself is probably another factor that distinguishes between the different IκBs. Mathematical models and experimental data indicated that activation kinetics of NF-κB is affected by the timing and duration of the IKK activity (97). Furthermore, the affinity between IKK and the different IκBs correlates with their degradation rate, implicating IKK as the main factor that shapes the differential kinetics (98). Nevertheless, it is unclear whether the affinity is sufficiently high to explain how IKK finds its IκB target within the degradation time frame. In an analogous signaling system, such as the Wnt signaling cascade, the substrate is brought to the kinase through scaffolding proteins. Thus, APC and axin scaffolds adhere both to the substrate β-catenin as well as to the kinases CKIα and GSK3 and promote rapid phosphorylation and subsequent degradation of β-catenin (99). It will be of interest to search for a similarly functioning scaffold in the NF-κB pathway.

Another factor that might affect the degradation kinetics is differential rates of ubiquitination for different IκBs. Since all the IκBs share the same DSG degron, it is less plausible that the recognition by β-TrCP is discriminative, but perhaps other parts of the IκB structure can influence the processivity of ubiquitination, a major factor limiting the rate of degradation (100). The processivity of ubiquitination is influenced by the affinity to the E3 ligase and by the associated activity of deubiquitinating enzymes (DUBs) (100). Deubiquitination would reverse the rate of ubiquitination and thus could reduce both the basal and signal-dependent ubiquitination of several proteins in NF-κB signaling, including IκBs. CYLD and A20 are the two major DUBs in the NF-κB pathway (101), but apparently, none targets IκBs (102). No specific DUB has been found to control the degradation of IκB, but considering equivalent signaling systems [e.g. the DNA damage response (103, 104)], it may exist, thus providing another important regulatory aspect to NF-κB signaling (*Fig.* 3).

Differences in the degradation kinetics of the IκBs are in line with their non-redundant roles in regulating the NF-κB response. A good example is the computational model of Hoffmann *et al.* (105, 106). Using a set of mouse embryonic fibroblasts with individually deleted IκB, it was shown that IκBα is unmatched in inducing a rapid, strong negative feedback regulation, resulting in an oscillatory NF-κB activation

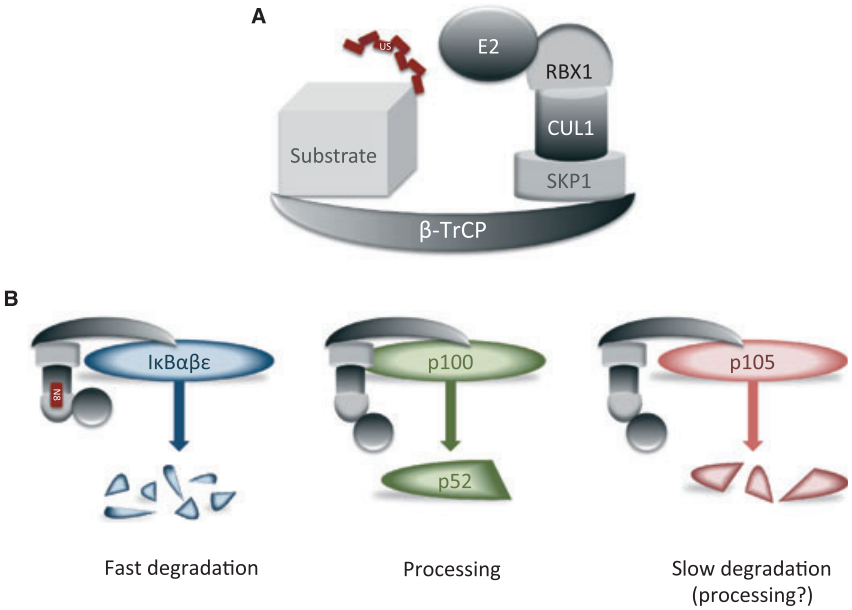

***Fig. 2.* Differential fates of substrates undergoing β-TrCP-mediated ubiquitination.** (A). Schematic structure of the $SCF^{\beta\text{-TrCP}}$ complex made of 5 components: The F-box protein β-TrCP, Skp1, Cul1, Rbx1/Roc1 and an E2 enzyme (e.g. UbcH5) positioned next to its substrate undergoing poly-ubiquitination. (B). A hypothetical model, attributing the rate of ubiquitination to Cul1 neddylation (Nedd8 conjugation). Whereas neddylation facilitates long-chain ubiquitination (175) and degradation of the 'professional IκBs' (α,β,ε), its absence may yield short-chain or mono-ubiquitination, which is possibly sufficient for signaling proteasomal processing (118) or slow degradation of a substrate.

profile. IκBβ and particularly IκBε respond more slowly to IKK activation and out of phase with IκBα, thus acting to dampen the evolving oscillations of the NF-κB response (105). The interplay between the different IκBs dictates the onset and termination of NF-κB activation, allowing a relatively stable NF-κB response during long-term stimulation. Consistent with the central role of IκBα, deletion of IκBα leads to a constitutive NF-κB activation and postnatal lethality (106). In contrast, deletion of IκBβ or IκBε in mice generate a far less striking phenotype indicating that other IκBs, possibly including the Ankyrin-containing Rel proteins (see below), can compensate for their loss (105, 107, 109). Notably, when IκBβ was expressed under the promoter of IκBα, it provided full compensation for the absence of IκBα (110). This finding highlighted the importance of the transcriptional regulation of IκBα, rather than its biochemical properties in controlling NF-κB activity.

In addition to their distinctive degradation kinetics, the different IκBs show dissimilar quantitative contributions to the NF-κB response. In particular, the relative stoichiometry of association of each IκB and NF-κB is not known. There is, however, a negative correlation between the relative concentration of nuclear NF-κB and the total cellular IκBα levels, indicating a major role of IκBα in controlling the NF-κB localization (111). However, as interaction of individual IκBs with NF-κB is mutually exclusive and the different IκBs have distinctive influence on NF-κB translocation into the nucleus, variations in the abundance of the different IκBs may influence NF-κB activation indirectly, via hindering the association of NF-κB with IκBα. Thus, as IκBβ competes with IκBα for NF-κB binding and is far less effective in inhibiting NF-κB activation, ablation of IκBβ renders mice more susceptible to LPS endotoxemia (94). It will therefore be interesting to study NF-κB localization with respect to the relative concentrations of the various IκBs and to incorporate these figures into the mathematical model of NF-κB activation.

NF-κB1 p105 and NF-κB2 p100 also function as IκBs (112, 113), but exactly how they integrate into the canonical NF-κB activation pathway is mostly unresolved (4). Their primary function is to serve as precursors for the mature NF-κB proteins p52 and p50, a function that is regulated by proteasomal processing (114) (Fig. 2). The ankyrin repeat structure of p100 and p105 binds NF-κB/Rel proteins similarly to the homologous region of the 'professional IκBs' (α, β, and ε), and thereby may fulfill an IκB function, either cytoplasmic retention of the bound Rel protein, or inhibition of DNA binding. To effectively function as IκBs, the precursor bound NF-κB must, under certain conditions, dissociate, allowing the associated Rel molecule to enter the nucleus and induce transcriptional activity. One way for the precursors to release their bound NF-κB is during processing. Signal-facilitated processing would then enhance NF-κB release; indeed, the

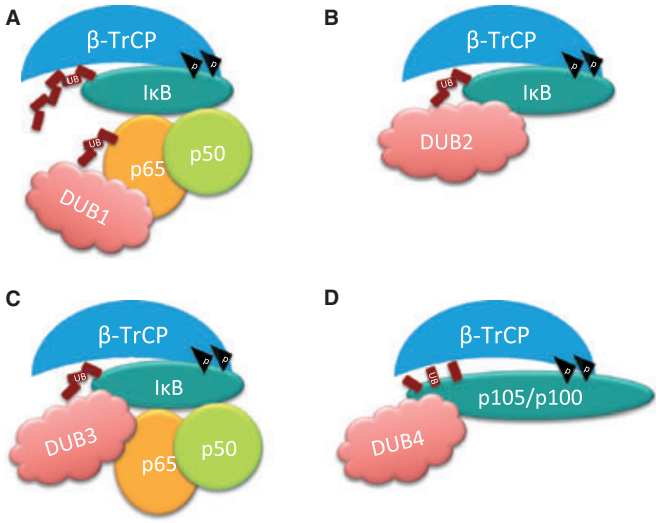

Fig. 3. A hypothetical model showing putative effects of deubiquitinating enzymes (DUBs) in post-IKK NF-κB signaling. (A). An associated DUB1 maintains the stability of NF-κB within a complex with IκB, by specifically reversing NF-κB ubiquitination. This results in too short ubiquitin chains on NF-κB and proteasome-recognizable long chains on IκB (100). (B). DUB2, an early-induced NF-κB target gene, interacts with the 'naked' (NF-κB–unbound) newly synthesized IκB, preventing long-chain ubiquitination and degradation, despite IKK phosphorylation. (C). DUB3, a product of a late-induced NF-κB target gene reduces the ubiquitin chain length of IκB, thereby slowing proteasomal degradation (100) and contributes to dampening the NF-κB response despite IKK prolonged activity (97). (D). DUB4 interacts with the NF-κB precursors p105 and p100, trimming their ubiquitin chains down to ubiquitin monomers, which may be sufficient for processing (118), or for slow but not rapid degradation.

processing of p100 and p105 are induced by cell stimulation. p100 is phosphorylated by IKKα, the latter is recruited and activated by NF-κB-inducing kinase (NIK) as part of the non-canonical NF-κB signaling pathway (115, 116). This precursor phosphorylation promotes the binding of β-TrCP, polyubiquitination, and proteasomal processing, generating an active IκB-free p52 (58). p105, however, is primarily processed constitutively (56, 117), but the rate of processing is enhanced in stimulated cells (113). In contrast to p100, p105 processing appears to be mediated by a different E3 than β-TrCP, which has yet to be identified. This E3 is likely to modify p105 by mono-ubiquitination rather than polyubiquitination mediated by β-TrCP (118).

A study in MEFs lacking all 'professional' IκBs (IκBα$^{-/-}$, IκBβ$^{-/-}$, and IκBε$^{-/-}$) provided evidence that p100 functions as a canonical NF-κB inhibitor upon lymphotoxin β receptor (LTβR) stimulation (109). Following TNF stimulation, the fraction of p100-bound, dissociable p65 was found to be increased three to fourfold, creating a specific time window for an IκB function of p100 in WT cells, indicating a possible synergism between LTβR and TNF in canonical NF-κB activation. It remains to be seen whether this synergism is evident in cells other than MEFs and if the overall stoichiometry of p100-bound NF-κB is significantly changed following canonical stimulation. Furthermore, transcriptional synergism between LTβR and TNF via the p100 IκB function should be distinguished from other mechanisms, such as IKK activation by LTβR (119).

While p105 processing would suppress its IκB function, augmenting NF-κB activation (120), and is also compliant with canonical NF-κB signaling, a fraction of p105 is targeted for complete degradation following certain cell stimuli, such as TNF (55). Overexpressed p105 was found to sequester c-Rel and p65 in the cytoplasm of Cos-7 and CV-1 cells (112). Endogenous p105 and p100 were shown to associate with c-Rel and p65 in Jurkat cells (113) and in WT MEFs *in vivo* (109). Nevertheless, the physiological significance of the IκB function of the precursors has not been fully addressed and the stoichiometry of the NF-κB fraction that is bound to these proteins is unknown. The Rel fraction that is bound to the precursors might be different from cell to cell and be subject to varying cellular conditions; hence, its quantification might help in understanding the IκB role played by the NF-κB precursors.

In vivo assessment of the IκB role of p100 and p105 is difficult, as most genetic manipulations will also influence p50 and p52. Modifying the precursor by means of site-directed mutagenesis should provide a means to selectively interrogate precursor protein IκB-like functions. This was successfully exercised with p105 in T cells, showing that substitution of the modifiable serine residues that distinctively control p105 stability resulted in a moderate 'IκB super-repressor'-like

molecule, affecting T-cell development (121). However, again the stoichiometry of p105-bound NF-κB is non-physiological, thus not necessarily attesting to a physiological IκB function of p105 in T cells. Whereas a heterozygous mutation that substitutes Ser32 of human IκBα is sufficient to cause T-cell immunodeficiency and ectodermal dysplasia (122, 123), similar mutations in the precursor genes *NFKB1* and *NFKB2* have yet to be found. Conceivably, better understanding of the true IκB role of p100 or p105 requires quantitative and kinetic studies, in comparison to the 'professional' IκBs, preferentially under physiologic conditions, which may be best achieved with the help of mathematical modeling (106). Only then may we understand the impact of each IκB in physiological NF-κB response.

Temporal aspects of IκB degradation

Temporal regulation is fundamental for any signaling activity and is particularly important for limiting a response, such as NF-κB activation, which if unrestrained could be very harmful for the organism. A major factor controlling the lifetime of the NF-κB response is a negative feedback loop, based on NF-κB-stimulated IκB synthesis (124). Following a near-complete signal-induced degradation of IκB, newly synthesized IκBα enters the nucleus and detached NF-κB away from the chromatin, back to the cytoplasm (125). This process must allow a sufficient time window of NF-κB activity to induce transcriptional activity and at the same time avoid extending the transcriptional activity beyond the necessary time (105). While bound to chromatin, NF-κB undergoes various modifications, which may prevent premature termination of the transcriptional activity by IκB. Thus, RelA acetylation in the nucleus has been reported to prohibit its interaction with IκB, a process reversed by HDAC3-mediated deacetylation, following which, NF-κB is evicted from the chromatin by the newly synthesized IκBα. A temporally coordinated acetylation-deacetylation cycle was shown to maintain NF-κB activation for 45 min (126). Indeed, following TNF stimulus, NF-κB is activated for approximately 45 min (105), while IκBα is transcribed as early as 15 min and peaks within 1 h (127).

An outstanding issue concerns the coordination of IκBα degradation and resynthesis with IKK activity. It has been reported that IKK activity peaks after 15 min and is maintained for more than 1 h (96), during which period all the newly synthesized IκBα would conceivably be phosphorylated by IKK and subsequently degraded. How then would IκB terminate NF-κB activation in the presence of a continuously active IKK? One possibility is that IKK phosphorylates the NF-κB-bound IκB far more efficiently than free IκB (128), explaining how the *de novo* synthesized free IκB can accumulate in IKK-active cells. This presumption, however, is not compatible with the finding that a major fraction of the newly synthesized IκB appears phosphorylated (129), suggesting that phosphorylation would not be the rate limiting step in the destruction of this newly synthesized molecule. Another possibility is inefficient ubiquitination of unbound IκB by β-TrCP. Indeed, the ubiquitination and degradation of IκBα is distinct between the NF-κB-bound and the unbound molecule (28, 130) (*Fig.* 3). Unlike many SCF substrates, most lysine residues of IκB are not targeted by β-TrCP *in vivo* or *in vitro* when bound to NF-κB; the only relevant ubiquitination residues are Lys 21 and 22 of IκBα (131). Lys21 is also a target for sumoylation, a modification that could antagonize ubiquitination, and thus would spare the newly synthesized IκB. Nevertheless, sumoylation seems to happen only in the nucleus (132) and would not explain why the newly synthesized IκBα is not phosphorylated and degraded in the cytoplasm. Likewise, it has been shown that overexpressed SUMO-1 blocked TNF and interleukin-1 (IL-1)-dependent ubiquitination and degradation of IκBα in Cos7 cells (133), but S32/36-phosphorylated IκBα was resistant to sumoylation, suggesting that this modification would again be relevant only prior to IκB phosphorylation by IKK.

The issue of sparing newly synthesized Iκβα is further underscored by finding that an unbound IκB could also be targeted by E3s that do not require IKK phosphorylation (28, 130) and thus be degraded as efficiently as the phosphorylated IκB by β-TrCP (130). IκBα can also be phosphorylated independently of a signal on its C-terminus by the kinase CK2 (134) or in response to a noncanonical stimulus, such as ultraviolet radiation (UV) (135). Depletion of the CK2β subunit or mutations at the phosphorylated residues in the C-terminal fragment of IκBα abrogated the NF-κB response following UV (135). Likewise, in response to doxorubicin-induced DNA damage, IκBα undergoes proteasomal degradation, both in WT and in IKKα$^{-/-}$, IKKβ$^{-/-}$ MEFs (136). Assuming that all these data are physiologically relevant, it further implies that we do not understand how newly synthesized IκB, whether phosphorylated or not, escapes degradation. If the IκB-mediated chromatin-eviction of NF-κB is indeed a key mechanism of NF-κB termination, there must be a mechanism to shield the newly synthesized IκBα from the UPS. One possibility is association with a deubiquitinase (DUB), which will reverse ubiquitination and thus preclude IκB degradation (*Fig.* 3).

Pros and cons of targeting IκB degradation in cancer and inflammation

Aberrant NF-κB regulation has been implicated in multiple chronic diseases, from neurodegenerative diseases to autoimmune diseases and cancer (137–139). Experimental mouse models of NF-κB inhibition are primarily based on two strategies, targeting IKK (140, 141) and blocking NF-κB activation by overexpression of a non-degradable inhibitor (39, 142). Of the two strategies, only IKK inhibition would be available for human studies, yet, to the best of our knowledge, whereas quite a few IKK inhibitors have been developed and found to exhibit anti-tumor and anti-inflammatory effects (143–146), as of the end of 2011, no such drug has been clinically approved. This limited success of IKK inhibitors has prompted the development of alternative blockers including inhibitors of the ubiquitin-proteasome system (UPS) (147–149). In particular, combinations of bortezomib, a proteasome inhibitor, and various other new anti-cancer drugs, such as HDAC inhibitors, have already found their way into advanced clinical studies.

Given their central role in regulating NF-κB activation, proteins involved in ubiquitination and degradation of the IκBs are attractive targets for drug development (101). The successful treatment of a major hematological malignancy using a proteasome inhibitor has been the proof of concept for exploiting the ubiquitin-proteasome system as therapeutic target (150). Bortezomib (formerly known as PS-341), a boronic acid dipeptide that binds directly with and inhibits the proteasome enzymatic complex, shows significant therapeutic activity in the treatment of advanced multiple myeloma (MM) (151) and was approved for MM treatment by the Food and Drug Administration in 2003. MM is a particularly attractive target for NF-κB inhibition, as it often carries a variety of NF-κB pathway mutations, most of which are affecting the pathway upstream of IκB degradation (152–156). The Bortezomib effects on cells are partly mediated through NF-κB inhibition (151, 157), resulting in apoptosis, decreased angiogenic cytokine expression, and inhibition of tumor cell adhesion to stroma. Bortezomib has also been used in combination therapy; the most extensively studied is a combination of bortezomib and vorinostat (HDAC inhibitor). A phase III trial (NCT00773747) of this pair is being conducted in patients with relapsed and/or refractory MM following a very good response in a third of the patients, which in earlier clinical trials failed bortezomib monotherapy (158).

Continuous proteasomal inhibition may be toxic, for which reason bortezomib cannot be administered more than twice weekly, with proteasome inhibition lasting approximately 24 h after each injection (159). Blocking IκB ubiquitination may therefore represent a safer alternative to proteasomal inhibition and may be particularly attractive for cancer therapy (35). One could imagine blocking a subset of relevant E3s or even a particular E3. An interesting new addition to the UPS inhibitor arsenal is the NEDD8 activating enzyme (NAE) inhibitor MLN4924, which blocks NAE and NF-κB signaling in a primary diffuse large B-cell lymphoma (DLBCL), resulting in tumor regression (160). NAE activates Cul-1 Neddylation, which is required for $SCF^{\beta\text{-}TrCP}$ (*Fig.* 2). β-TrCP by itself, is an attractive target for NF-κB inhibition; its inhibition would result in IκB stabilization. The effectiveness of blocking IκB degradation for ameliorating inflammatory diseases and cancer has been established in numerous experiments, both *in vitro* and *in vivo*, mostly using a dominant IκB super-repressor, which cannot be phosphorylated and ubiquitinated but maintains its NF-κB inhibitory capacity (39, 161). One *in vivo* example is the expression of such an inhibitor in liver cells resulting in inhibition of tumor progression in a murine model of hepatitis-associated cancer (40).

A clinically relevant method for that purpose is to suppress the activity of β-TrCP, or more specifically, to block the interaction of β-TrCP with IκB. Cell-penetrating IκB phosphopeptides, which compete with endogenous IκB for binding to β-TrCP have been used for this purpose in cell lines (28), showing NF-κB inhibition, but would probably not work *in vivo* for two main reasons: they would likely be degraded rapidly by cellular proteases and the phosphate moieties will hinder their cell penetration. Inhibitory peptide stability may be however improved by a technique called backbone cyclization. This has been shown to increase the stability and selectivity of inhibitory peptides. Backbone cyclic peptides with conformational diversity have produced bioactive peptides with improved pharmaceutical properties, metabolic stability, and enhanced intestinal permeability (162). Cyclic IκB phosphopeptides were found to block effectively IκB ubiquitination (163). It might also be possible to develop small-molecule inhibitors that structurally mimic the ligase recognition motif (peptidomimetics) or ones that specifically inactivate $SCF^{\beta\text{-}TrCP}$ (through allosteric inhibition or disassembly).

An obvious drawback of targeting β-TrCP for NF-κB inhibition is the many $SCF^{\beta\text{-}TrCP}$ substrates, other than IκBs (72). A critical issue is potential deleterious effects of the accumulation of various $SCF^{\beta\text{-}TrCP}$ substrates, particularly if it is the only E3 that contributes to the degradation of any given substrate. A serious concern, for example, is stabilization of β-catenin,

an important factor in colorectal tumorigenesis and other cancer types (164). It has however been shown that a distinct E3 complex containing the Siah RING finger protein recognizes and promotes the degradation of β-catenin, independently of $SCF^{\beta\text{-TrCP}}$ (165, 166). A useful way of monitoring the feasibility of systemic β-TrCP-deficiency could be its ablation in the adult mouse, where NF-κB inhibition would not be immediately deleterious, unless the mouse is exposed to a pathogen or another hazard (39). β-TrCP is encoded by two mammalian paralogs, which apparently have many redundant functions, including the ubiquitination of the IκBs and β-catenin. Male β-TrCP1 (BTRC)-deficient mice have only moderate impairment in spermatogenesis and reduced fertility without other signs of gross tissue abnormalities and illness, probably due to the compensatory effect of β-TrCP2 (Fbxw11) (61). Indeed, deletion of the two β-TrCP paralogs, even conditionally at the adult mouse, is lethal in a matter of days (N. Kanarek, unpublished data). Considering the apparent redundancy of the two isoforms on top of the critical function of β-TrCP in many adult tissues, Kanarek *et al.* generated a mouse strain carrying an inducible shβ-TrCP2 transgene and crossed it to the β-TrCP1 knockout mouse. By achieving transient, incomplete ablation of β-TrCP2 on a full β-TrCP1 deleted background, this mouse model overcame some of the limitations of traditional conditional ablation studies, simulating a drug inhibitory effect. Surprisingly, this model taught us that achieving a very high-level systemic inhibition of β-TrCP is still compatible with the well-being of treated mice, indicating a considerable physiologic robustness of cellular protein turnover. Conceivably, cancer cells, unlike normal cells, are far more vulnerable to deregulation of protein turnover (167); an example is multiple myeloma cells succumbing to partial intermittent proteasomal inhibition (151). For certain tissues however, the distinction between normal and malignant cells may not always hold true and if a system relies on dynamic protein turnover, even deregulation of a single β-TrCP substrate, like Snail1 in the testis, may greatly compromise normal homeostasis (38). If, however, the prevailing mild effect of transient, incomplete β-TrCP inhibition in humans would be similarly to mice, temporary $SCF^{\beta\text{-TrCP}}$ inhibition may be systemically tolerable, providing means of specifically affecting tumor cells.

If β-TrCP inhibition is indeed a technically feasible goal, systemic inhibition of NF-κB is not without risk due to the crucial role of the transcription factor in regulating immune responses (168). This is of a particularly important consideration when NF-κB inhibition is attempted in combination with standard chemotherapy, which by itself compromises the immune system and exacerbates tissue damage (39). One should then be cautious with those types of cancer in which NF-κB activation could be a homeostatic switch, possibly limiting genotoxic damage (169). Moreover, NF-κB inhibition may in fact be a 'double-edged sword', as in the presence of a carcinogen, and perhaps even under chemotherapeutic poisoning, NF-κB inhibition may facilitate rather than prevent tumor development (170). This scenario, so far apparent only in experimental cancer systems, should be taken into account when considering an NF-κB blocking therapeutic regimen and possibly handled with a drug combination that will rebalance the adverse effects of NF-κB inhibition. Thus, it has been shown that NF-κB inhibition promotes enhanced IL-1β secretion and neutrophilia (171), which are hallmarks of certain serious human inflammatory syndromes (172–174). One might then consider a combination therapy of NF-κB inhibition in conjunction with anti-IL-1 therapy, which together may reverse this possible proinflammatory effect, although at a high cost of increased susceptibility to infections.

Perspectives: conclusions and outstanding questions

Inducible IκB degradation is a paradigm of targeting a regulatory protein by the ubiquitin proteasome system as an integral step in the activation of a signaling pathway. A key feature of this process is the coupling of two protein modification events, signal-induced phosphorylation and the subsequent ubiquitination of a protein. Phosphorylation appears to be the rate-limiting step in the IκB degradation process, and its whole purpose is to create a specific degron, based on a conserved sequence motif. Following this step, the degron is specifically recognized by β-TrCP, a subunit of a Cullin-RING E3 ligase (CRL), and rapidly targeted for ubiquitination and proteasomal degradation. Ubiquitination of IκBα by β-TrCP is one of the most efficient inducible protein-modification effects noted in signaling cascades, as virtually all the IκBα content of a cell is converted within minutes to highly poly-ubiquitinated species and degraded. Many details of this signaling step have been investigated in detail, providing a great deal of structure–function relationship. However, we do not understand certain distinctions of this process, such as the basis for the apparent very high processivity of IκBα ubiquitination, which is not a general feature of other β-TrCP substrates both within the NF-κB pathway and beyond.

This is by no means the only remaining mystery surrounding IκB degradation and among the outstanding questions are the following:

1. Where in the cell is the ubiquitination and degradation of IκB occurring and is it linked to IKK phosphorylation similarly to the β-catenin ubiquitination process, which is regulated by the same E3? In other words, is the phosphorylation–ubiquitination process of IκB coordinated in a scaffold-based destruction complex?

2. Is IκB ubiquitination reversible; is there a physiological process of IκB deubiquitination and does a specific deubiquitinating (DUB) enzyme regulate this putative reaction? A possibly related question is how is the NF-κB-transcribed, newly synthesized IκB protected from degradation as long as IKK is still activated? Could the newly synthesized IκB, in contrast to the one in complex with NF-κB be a ready DUB substrate, which would reverse its ubiquitination and thus protect it from degradation?

3. What determines the kinetics of degradation of the different IκBs? Is it the rate of phosphorylation, ubiquitination or perhaps even the proteasomal action? Are there specific proteasome adapters molecules that regulate the degradation of the different IκBs

4. How is NF-κB that is in complex with IκB protected from ubiquitination and degradation? This is matter of relevance, especially considering the known capacity of β-TrCP to mediate the destruction of a substrate-associated protein, such as HIV-Vpu1-associated CD4.

5. How frequently are mutations in the IκBs or their destruction machinery encountered in human disease?

Whereas, solving these remaining questions is mostly a matter of basic research, there is a possibility that this research will also facilitate a relevant translational research, with the goal of taming NF-κB whenever it exceeds physiological requirements. In particular, detailed structure-function studies of β-TrCP may promote the development of small molecule or peptidomimetic inhibitors of β-TrCP. While there is still only fragmentary data on the effects of systemic inhibition of β-TrCP, preliminary studies indicate there may be an exploitable therapeutic window for β-TrCP blocking reagents as a means of NF-κB inhibition in cancer and inflammatory diseases. As of today, targeting the ubiquitin system, in contrast to the proteasome, has not found its way to the clinic. Therapeutic regulation of IκB degradation is therefore an appreciable challenge, but quite possibly a worthy effort.

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