CRL3IBTK Regulates the Tumor Suppressor Pdcd4 through Ubiquitylation Coupled to Proteasomal Degradation*

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Background: IBtkα is an uncharacterized protein belonging to the family of BTB proteins. Results: IBtkα is the substrate receptor for a Cullin3-dependent ubiquitin ligase promoting ubiquitylation and proteasomal degradation of Pdcd4. Conclusion: By regulating Pdcd4 stability, IBtkα can modulate the translation of specific mRNAs under different cellular conditions. Significance: The identification of new players in the ubiquitin/proteasome pathways contributes to a better understanding of protein homeostasis.

The human inhibitor of Bruton’s tyrosine kinase isoform α (IBtkα) is a BTB protein encoded by the IBTK gene, which maps to chromosomal locus 6q14.1, a mutational hot spot in lymphoproliferative disorders. Here, we demonstrate that IBtkα forms a CRL3IBTK complex promoting its self-ubiquitylation. We identified the tumor suppressor Pdcd4 as IBtkα interactor and ubiquitylation substrate of CRL3IBTK for proteasomal degradation. Serum-induced degradation of Pdcd4 required both IBtkα and Cul3, indicating that CRL3IBTK regulated the Pdcd4 stability in serum signaling. By promoting Pdcd4 degradation, IBtkα counteracted the suppressive effect of Pdcd4 on translation of reporter luciferase mRNAs with stem-loop structured or unstructured 5’-UTR. IBtkα depletion by RNAi caused Pdcd4 accumulation and decreased the translation of Bcl-xL mRNA, a well known target of Pdcd4 repression. By characterizing CRL3IBTK as a novel ubiquitin ligase, this study provides new insights into regulatory mechanisms of cellular pathways, such as the Pdcd4-dependent translation of mRNAs.

Protein ubiquitylation is an essential process for proteasome-mediated degradation or signalosome recruitment of proteins in response to extracellular stimuli (1, 2). Ubiquitylation occurs through three sequential steps, where ubiquitin (Ub)5 is first activated by the Ub-activation enzyme (E1) and then transferred to the Ub-conjugating enzyme (E2) and finally attached to the protein substrate by the Ub ligase (E3) (1). The largest family of E3 ligases consists of multisubunit complexes, including the scaffold proteins of the Cullin (Cul) family, which are named Cul-RING ligases (CRLs) (3). CRLs are composed of three major elements: (i) Cul protein; (ii) the catalytic module, composed of a RING finger protein (RBX1 or RBX2), which interacts with the C-terminal domain of Cul and recruits the Ub-conjugating enzyme (E2); (iii) the substrate recognition module, which interacts with the N-terminal domain of Cul and places the substrate in close proximity of the catalytic module, thus facilitating the Ub transfer (4). NEDD8 covalent modification of the C-terminal domain of Cul is additionally required to induce the conformational changes of CRL structure for bringing the substrate and E2-ubiquitin into juxtaposition (5).

The best characterized CRL is the Skp1/Cul1/F-Box (SCF) complex, where Cul1 binds to Rbx1 and to the adaptor protein Skp1, which in turn associates with the F-box protein responsible for substrate recruitment to the SCF complex (6–8). A similar molecular organization is observed in Cul3-based CRL (CRL3), which consists of Cul3, Rbx1, and the substrate-specific adaptor with one or more Bric-a-brac, Tramtrack, and Broad complex/Pox virus and zinc finger (BTB/POZ) domain (9, 10), hereafter referred to as the BTB domain. BTB proteins bind to Cul3 via the BTB domain (11) and determine the substrate specificity of the ubiquitin ligase complex through an additional protein–protein interaction domain, including the MATH (meprin and TRAF homology) domain, Kelch (KLH) repeats, zinc fingers, or ankyrins (12). Hence, the BTB proteins

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5 The abbreviations used are: Ub, ubiquitin; Cul, Cullin; CRL, Cul-RING ligase; SCF, Skp1/Cul1/F-Box; CRL3, Cul3-RING ligase; BTB, Bric-a-brac, Tramtrack, and Broad Complex; POZ, pox virus and zinc finger; KLH, Kelch; RCC1, regulator of chromosome condensation 1; IP, immunoprecipitation; WB, Western Blot; RBD, RNA-binding domain; aa, amino acids; CHX, cycloheximide; RIPA, radiolabeled protein precipitation assay.
incorporate the features of the Skp1/F-box dimer of CRL1 within a single polypeptide (11, 13–15).

The BTB domain is evolutionarily conserved and mediates a variety of biological processes, such as transcriptional regulation, ion channel assembly, cytoskeleton dynamics, apoptosis, and protein ubiquitylation (3, 8). In *Schizosaccharomyces pombe*, three BTB proteins (Btb1, Btb2, and Btb3) have been identified, which function as substrate receptors of Pcu3, the yeast Cul3 orthologue (14). The human genome encodes nearly 200 BTB proteins (16), with only a small subset having been characterized as substrate adaptors of CRL3 (12). A common structural feature of BTB proteins of CRL3 is the presence of a paired helical structure, named the 3-box motif, which consists of a two-helix extension of the BTB domain that is critical for high affinity interaction with Cul3 (12, 17). A well known substrate receptor of CRL3 is the BTB/Kelch protein Keap1, which promotes the ubiquitylation coupled to proteasomal degradation of Nrf2, a transcriptional factor involved in oxidative stress response (18–21). Other substrates of mammalian Cul3 include Dishevelled of Wnt signaling, Aurora B kinase, cyclin E1, RhoA, WNK kinase isoforms, the GluR6 kainate receptor, Daxx, RhoBTB2, topoisomerase 1, and Ci (12). The relevance of BTB proteins in human pathology has been highlighted by mutations of BTB proteins that are responsible for diseases, such as gigaxonin in giant axonal neuropathy (22), KLHL9 in autosomal dominant distal myopathy (23), autosomal dominant retinitis pigmentosa (22), and Gordon’s hypertension syndrome (24). From this perspective, the functional characterization of novel BTB proteins is relevant for substrate specificity of CRL3 and their implication in human diseases.

The human inhibitor of Bruton’s tyrosine kinase (*IBTK*) gene maps at the 6q14.1 cytogenetic location, which is a region of recurrent chromosomal aberrations in lymphoproliferative disorders (25). The *IBTK* gene has a complex organization because it expresses three coding transcripts for IBtkα, β, and γ protein isoforms and additional non-coding transcripts, including the pre-miRNA IBTK (26, 27). IBtkγ is the first identified 26-kDa protein isoform that acts as an inhibitor of Btk in B-cell receptor signaling (25, 28). IBtkα is the most highly and ubiquitously expressed protein isoform with a molecular mass of 150 kDa and has not been functionally characterized. IBtkα harbors multiple domains, including two ankyrin repeats at the N terminus, followed by three regulator of chromosome condensation 1 (RCC1) domains, two separated BTB domains, and a large C-terminal region of about 500 amino acid residues with no recognizable motifs (26). IBtkα is structurally related to S. pombe Btb1, a substrate receptor of the yeast Pcu3 (Cul3)-based ubiquitin ligase complex (11, 14). Based on the structural homology of IBtkα with Btb1, in this study, we addressed the question of whether IBtkα was a substrate receptor of CRL3-rerecruiting proteins for ubiquitylation and subsequent degradation by the proteasome.

**Experimental Procedures**

**Plasmids, siRNAs, Lentiviruses, and Antibodies**—pCMV6-IBtkα-FLAG (RC218657, IBtkα 1–1352) and pCMV6-XL5-Pdc4d were from OriGene Technologies, Inc. (Rockville, MD). pcDNA3-Myc-Cul3 (plasmid 19893), pcDNA3-Myc-Cul3ΔN41 (plasmid 21590), pcDNA3-DN-hCul3-FLAG (plasmid 15820), and pcDNA3-HA2-Rbx1 (ROC1) (plasmid 19897) were from AddGene (Cambridge, MA). The pCMV-LUC and pCMV-SL-LUC plasmids were a kind gift from Dr. Hsin-Sheng Yang (Graduate Center for Toxicology, University of Kentucky, Lexington, KY). The proarykotic expression vector of Pdc4d wild type and mutants fused to GST (GST-Pdc4d-WT, GST-Pdc4dDRBD, or GST-Pdc4dRDStop) were a kind gift of Dr. K. H. Klemmner (Westfälische-Wilhelms-Universität Munster).

GenScript Corp. (Piscataway, NJ) generated the following eukaryotic expression vectors of IBtkα mutants: pCMV6-IBtkαΔC-FLAG (aa 1–890), pCMV6-IBtkαΔN-FLAG (aa 307–1352), pCMV6-IBtkαΔBTB-FLAG (deletion of aa 564–836), pcDNA3.1(+) Pdc4d-WT-HA, and pcDNA3.1(+) Pdc4d S67A/S71A/S76A.

ON-TARGET plus IBtkα siRNA, Cul3 siRNA, and control NO-TARGET siRNA were from GE Healthcare (Buckinghamshire, UK). ON-TARGET plus IBtkα siRNA includes a pool of siRNAs targeting the following sequences of IBtkα mRNA (NCBI reference sequence: XM_006715453.1): 2365–2474 (probe A002542), 2400–2638 (probe D6S1188E), 4113–4214 (probe D6S1109E), and 5776–5879 (probe D6S1882).

The lentiviral constructs expressing the shRNA against IBtkα or control non-targeting shRNA (TRCN0000082575 and SHC002, respectively) were from MISSION® (Sigma-Aldrich). The shRNA-IBtkα targets the 2077–2098 nucleotides of IBtkα mRNA (NCBI reference sequence: XM_006715453.1). Lentiviral particles were produced in HEK293T cells, as described previously (28, 29).

Mouse anti-Pdc4d, mouse anti-HA, mouse anti-GAPDH, and mouse IgG antibodies were from Santa Cruz Biotechnology, Inc. Rabbit anti-Pdc4d, anti-Myc, anti-Ub Lys48, and anti-Ub Lys63 were from Cell Signaling Technology. Anti-Cul3 antibody was from BD Biosciences. Anti-FLAG was from Sigma-Aldrich. Anti-IBtk antibody was from Bethyl Laboratories, Inc. (Montgomery, TX).

**Cell Lines, Transfection, and Treatments**—HeLa and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), supplemented with 10% heat-inactivated fetal calf serum, 2 mm l-glutamine and antibiotics (Life Technologies).

Cells were transfected with DNA using Lipofectamine 2000 (Life Technologies), according to the manufacturer’s protocol. For siRNA, cells (3 × 10⁶) were transfected with 100 nmol of the indicated siRNA. When required, cells were treated with the proteasome inhibitor MG132 (Sigma-Aldrich), or protein biosynthesis inhibitor cycloheximide (CHX) (Sigma-Aldrich).

**Cell Extracts, Immunoprecipitation (IP), and Western Blotting (WB)**—Cells were lysed in modified RIPA buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Igepal). For IP, cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Igepal, 0.5% sodium deoxycholate). Protein extraction was performed in the presence of protease inhibitor mixture (Roche Applied Science, Mannheim, Germany) and 2 mM N-ethylmaleimide (Sigma-Aldrich), using 1 ml of cold buffer for a 100-mm dish. Cell lysates were clarified by centrifugation at 14,000 × g for 10 min and then incubated overnight with the appropriate antibody, followed by a 2-h
incubation with G-protein beads (30 μl/sample) (GE Healthcare). The beads were washed five times with 1 ml of cold RIPA buffer and denatured for 10 min at 70°C in 25 μl of 2× NuPAGE sample buffer (Life Technologies). Protein samples were subjected to electrophoresis on NuPAGE 4–12% polyacrylamide gel (Life Technologies) or self-casted 6% polyacrylamide gel and then transferred onto a nitrocellulose membrane (GE Healthcare).

Mass Spectrometry—The pCMV6-IBtkα-FLAG plasmid and the corresponding empty vector were singularly transfected in HEK293T cells (24 μg of DNA/100-mm dish). Protein extracts (1.5 mg) from cells transfected with IBtkα-FLAG and empty vector were immunoprecipitated with anti-FLAG antibody (20 μg). Immunocomplexes were resolved by NuPAGE 4–12% SDS-PAGE, and gels were stained with colloidal Coomassie, as reported previously (30, 31). Protein bands were excised and subjected to in-gel tryptic digestion for mass spectrometry, according to Shevchenko et al. (32) and Käll et al. (33). Chromatography of tryptic peptides was performed on an Easy LC 1000 nanoscale liquid chromatography system (Thermo Fisher Scientific). The analytical nanoscale liquid chromatography column was a pulled fused silica capillary, 75-μm inner diameter, in-house packed to a length of 10 cm with 3-μm C18 silica particles from Dr. Maisch (Entringen, Germany). Peptide mixtures were loaded directly onto the analytical column at 500 nl/min. A binary gradient was used for peptide elution. Mobile phase A was 0.1% formic acid, 2% acetonitrile, whereas mobile phase B was 0.1% formic acid, 80% acetonitrile. Gradient elution was achieved at a 250 nl/min flow rate and ramped from 2% B to 45% B in 30 min. After 5 min at 100% B, the column was re-equilibrated at 2% B for 15 min before the following injection. MS detection was performed on a quadrupole-orbitrap mass spectrometer Q-Exactive (Thermo Fisher Scientific) operating in positive ion mode, with nano-electrospray ionization potential at 1800 V applied on the column front-end via a tee-piece. Data-dependent acquisition was performed using a report constructs pCMV-LUC and pCMV-SL-LUC. Cell lysates were analyzed for luciferase activity using the DualLuciferase assay kit (Promega), according to the manufacturer’s instructions.

Protein Synthesis—HeLa cells (6 × 10⁵) were seeded into 24-well plates and 24 h later were incubated for 1 h in the presence of 50 μCi of ³⁵S-labeled EasyTag Express protein labeling mix (PerkinElmer Life Sciences) in methionine-free medium supplemented with 10% dialyzed FCS. Then cells were lysed in RIPA buffer, and radiolabeled proteins were precipitated with trichloroacetic acid (TCA) on Whatman 3MM paper. The amount of radioactivity was determined by scintillation counting, and the counts were normalized to protein concentration.

Quantitative RT-PCR Analysis—Total RNA was extracted from cells with TRIzol reagent (Invitrogen). RNA aliquots (200 ng) were reverse transcribed with Random Examiners (Roche Applied Science) and Superscript III Reverse Transcriptase (Invitrogen), according to the manufacturer’s protocol. Real-time PCR was performed using iQ Green Super Mix (Bio-Rad) and carried out with the iCycler iQReal-Time detection system (Bio-Rad) under the following conditions: 95°C for 1 min and then 40 cycles at 94°C for 10 s and 60°C for 30 s. Primers were
as follows: IBtkα FW, 5′-GTCAGCCTCTCTTGGTGTGAT-3′; IBtkα REV, 5′-TGACATCTGGTTTGGGGGCCC-3′; Pdcd4 FW, 5′-CCATGGGCTTCAATAGTTCATGTT-3′; Pdcd4 REV, 5′-CCGCTTCTTCTCTTACACCCG-3′; Bel-X1 FW, 5′-GTGAGCTGGATCCGACTT-3′; Bel-X1 REV, 5′-GCTGGTCATTGGTCCCATAG-3′; GAPDH FW, 5′-CAGCCTCAAGATCATACAGCA-3′; GAPDH REV, 5′-TGTTGTTCAT-CAGTCTTCCA-3′. Relative mRNA levels were normalized to the GAPDH level.

Statistical Analysis—Statistical analysis was performed by a paired two-tailed Student’s t test. Differences were considered as statistically significant at the 95% level (p < 0.05).

Bioinformatics—Identity and similarity of human IBtkα and yeast Btb1 proteins were determined using the Clustal Omega program. Bioinformatics analysis for structural identification and representation of the 3-box motif of IBtkα (amino acids 861–901) was carried out using the Phyre2 computational program (35).

Results

IBtkα Is a Component of Cul3-dependent E3 Ligase—Clustal Omega multiple sequence alignment program-based bioinformatics analysis identified a significant homology of human IBtkα (UniProtKB Q9P2D0) and S. pombe Btb1 (UniProtKB O74881) with similarity and identity in amino acid positions of 30 and 20%, respectively (Fig. 1A). Domain organization is strictly conserved in the two proteins because they contain two ankyrin repeats at the N terminus followed by a tandem array of RCC1 domains and two separated BTB domains (Fig. 1B). Structural homology and fold recognition analysis carried out by the Phyre2 computational tool identified a 3-box motif placed at the C terminus of BTB2 domain, which significantly matched with the 3-box motif of different BTB proteins, such as SPOP, gigaxonin, KLH4, KLH11, and BTB6D (Table 1); the highest structural homology was with the 3-box motif of SPOP (confidence 98.2%) (Table 1). Because S. pombe Btb1 interacts with Pcu3p, the yeast orthologue of human Cul3, and is substrate receptor of Pcu3p(Cul3)-based ubiquitin ligase (11, 14), we speculated that IBtkα could be a substrate receptor of human CRL3.

We first investigated the in vivo interaction of IBtkα with Cul3. HEK293T cells were transfected with Myc-Cul3 in the presence or absence of IBtkα-FLAG, and 48 h later, cell extracts were immunoprecipitated with anti-FLAG or anti-Cul3 antibodies, followed by WB analysis of immunocomplexes. Myc-Cul3 was detected in the IBtkα-FLAG immunocomplex as two tandem protein bands with the expected molecular mass of about 90 kDa (Fig. 2A, lane 7), the slower band being the Cul3 neddylation form; as a control, Myc-Cul3 was not immunoprecipitated in Myc-Cul3-transfected cells in the absence of IBtkα-FLAG (Fig. 2A, lane 6). Similarly, IBtkα-FLAG was specifically detected in Myc-Cul3 immunocomplex (Fig. 2A, lane 10). As an additional experiment, the association of endogenous Cul3 with transfected IBtkα-FLAG or endogenous IBtkα was observed by IP of cell extracts with anti-IBtkα antibody and not control IgG (Fig. 2B, lanes 3 and 4). Altogether, these results indicated that IBtkα in vivo associated with Cul3.

Next, we mapped the amino acid sequence of IBtkα and Cul3 required for the association of the two proteins. The mutant IBtkαAC, lacking the C-terminal region including the 3-box motif (deletion of aa 891–1352; Fig. 2C), weakly bound to Myc-Cul3 (Fig. 2D, compare lanes 7 and 8). The mutant IBtkαN, lacking the ankyrin repeats and RCC1 domains (deletion of aa 1–307; Fig. 2C), was able to bind to Myc-Cul3 as efficiently as the wild type (Fig. 2D, compare lanes 7 and 9). Conversely, the mutant IBtkαDN, which lacked the BTB1 and BTB2 domains (deletion of aa 564–836; Fig. 2C) did not associate with Myc-Cul3 (Fig. 2D, compare lanes 7 and 10), which was consistent with the requirement of a BTB domain for binding to Cul3 (11). The N-terminal region of Cul3 was previously reported to interact with the BTB domain (20). Consistently, the mutant Cul3ΔN41 (deletion of 41 amino acids at the N terminus) (20) weakly coimmunoprecipitated with IBtkα-FLAG (Fig. 2E, compare lanes 9 and 10). Altogether, these results indicated that the IBtkα region, including the two BTB domains and 3-box motif, was required for the binding to the N terminus of Cul3.

To verify whether IBtkα was a component of a canonical CRL3, we tested whether the in vivo generated IBtkα-Cul3 complex included Rbx1, which is the CRL3 component recruiting the Ub-charged E2 enzyme (4). To this end, HEK293T cells were transfected with HA-Rbx1 in the presence or absence of IBtkα-FLAG and Myc-Cul3 expression vectors, and IBtkα-FLAG was immunoprecipitated with anti-FLAG antibody. HA-Rbx1 was detected in the IBtkα-FLAG immunocomplex in the presence of ectopically expressed Myc-Cul3 (Fig. 2F, lane 8), whereas it was undetected in the absence of IBtkα-FLAG and Myc-Cul3 (Fig. 2F, lanes 6 and 7). Overall, these results indicated that IBtkα was a component of a CRL3 complex including Cul3 and Rbx1.

IBtkα Is Autoubiquitylated through CRL3—A characteristic signature of most E3s is the ability to catalyze autoubiquitylation, which is usually assayed to confirm that proteins assemble into an active ubiquitin ligase complex (36). To assess whether IBtkα was ubiquitylated in vivo, HEK293T cells were transfected with the expression vector of IBtkα-FLAG and His-tagged ubiquitin, and the ubiquitylated proteins were pulled down and analyzed by WB with anti-FLAG antibody. Polyubiquitylated IBtkα-FLAG isoforms were observed (Fig. 3A, lane 2), which were slightly increased by overexpression of wild type Myc-Cul3 (Fig. 3A, lane 4) and almost abolished by overexpression of the dominant negative mutant DN-hCul3-FLAG (Fig. 3A, lane 5), which is devoid of the binding site for Rbx1, and thus inhibits the CRL3 catalytic activity (19). To analyze the pattern of IBtkα polyubiquitylation, HEK293T cells were transfected with the expression vector of IBtkα-FLAG or empty vector, and cell lysates were immunoprecipitated with anti-FLAG antibody and analyzed by WB with anti-Ub Lys48 or anti-Ub Lys63 antibodies. We observed Lys48 polyubiquitylation of IBtkα-FLAG that was strongly enhanced by cell treatment with the 26S proteasome inhibitor MG132 (Fig. 3B). No significant Lys63 ubiquitination of IBtkα-FLAG was detected (Fig. 3B, lanes 7 and 8). The half-life of IBtkα was determined in the presence or absence of MG132 by blocking ex novo protein synthesis with CHX. The IBtkα protein level slightly
decreased in the absence of MG132 with an estimated half-life of about 24 h, whereas it was unaffected in the presence of MG132 (Fig. 3, C and D). Overall, these results confirmed that IBtk was a component of a Cul3-dependent ubiquitin ligase complex, thereafter named CRL3IBTK, and underwent Cul3-dependent Lys48 polyubiquitylation coupled to proteasomal degradation.

IBtkα Physically Interacts with Pdcd4—To identify putative substrates of CRL3IBTK, we used a strategy based on immunopurification of ectopically expressed IBtk-H9251-FLAG in HEK293T cells, followed by mass spectrometry of IBtk-H9251-FLAG immunocomplex. We identified several IBtk-H9251 interactors that are involved in the ubiquitin-proteasome system pathway, including Cul3; UBA1 (ubiquitin-activating enzyme E1); ubiquitin;
and the classical CRL regulatory proteins NEDD8, CAND1, and subunit protein of the COP9 signalosome complex (Table 2). These findings supported a role of CRL3IBTK in the ubiquitin-proteasome system. We also identified the Pdcd4 (programmed cell death 4) protein, a well known tumor suppressor involved in several cellular processes (37). The association of endogenous Pdcd4 with transfected IBtkα-FLAG was proved by co-IP of cell extracts with anti-FLAG antibody (Fig. 4A). Co-IP of endogenous Pdcd4 and IBtkα proteins was observed using the anti-IBtkα antibody in the presence of the proteasome inhibitor MG132 (Fig. 4B).

### TABLE 1

| BTB proteins                                | % identity | Confidence | UniProt accession |
|----------------------------------------------|------------|------------|-------------------|
| Specle-type POZ protein                      | 41         | 98.2       | O43791            |
| Gigaxonin                                    | 15         | 96.8       | Q9H2C0            |
| Kelch repeat and BTB domain-containing protein 4 | 11         | 96.4       | Q9NVX7            |
| Kelch-like protein 11                        | 32         | 96.4       | Q9NV50            |
| BTR/POZ domain-containing protein 6          | 21         | 90.8       | Q96KE9            |

**FIGURE 2.** **IBtkα assembles within a CRL3 ubiquitin ligase complex in vivo.** A, association of ectopically expressed IBtkα and Cul3. HEK293T cells (3 × 10⁶) were transfected with IBtkα-FLAG (4 μg) or Myc-Cul3 (4 μg) or left untransfected. Forty-eight hours later, cell extracts (1 mg) were immunoprecipitated with the indicated antibodies and analyzed by WB. B, association of endogenous IBtkα and Cul3. HeLa cells (3 × 10⁶) were transfected with IBtkα-FLAG (4 μg) or left untransfected. The following steps were performed as indicated in A. C, schematic representation of IBtkα mutants used in this study. D, BTB domains of IBtkα are required for binding to Cul3. HEK293T cells (3 × 10⁶) were transfected with Myc-Cul3 (4 μg) and wild type or mutant IBtkα-FLAG (4 μg) or left untransfected. The following steps were performed as indicated in A. E, IBtkα binds to the amino terminus of Cul3 and Rbx1. HEK293T (3 × 10⁶) cells were transfected with IBtkα-FLAG (4 μg), Myc-Cul3 (4 μg), and HA-Rbx1 (4 μg). The following steps were performed as indicated in A. F, IBtkα generates a macromolecular complex with Cul3 and Rbx1. HEK293T (3 × 10⁶) cells were transfected with IBtkα-FLAG (4 μg), Myc-Cul3 (4 μg), and HA-Rbx1 (4 μg). The following steps were performed as indicated in A.
**Pdcd4 Regulation by CRL3IBTK**

**FIGURE 3.** IBtkα undergoes Cul3-dependent Lys48 polyubiquitylation and proteasomal degradation. A, Cul3 mediates the polyubiquitylation of IBtkα. HEK293T cells (3 × 10⁶) were transfected with His-tagged ubiquitin (His-Ub; 12 μg), with or without IBtkα-FLAG (4 μg), Myc-Cul3 (4 μg), or DN-hCul3-FLAG (4 μg) expression vectors. Forty-eight hours after transfection, cells were treated with MG132 (20 μM) for 4 h before lysis. Cell extracts were pulled down with GST-Pdcd4-WT (Fig. 4A, lane 4) or the corresponding empty vector (4 μg), and 48 h later, cells were treated with MG132 (20 μM) for 4 h before lysis. Cell extracts were immunoprecipitated with anti-FLAG antibody and resolved by 6% SDS-PAGE, followed by immunoblotting with anti-UP Lys48- or anti-UP Lys63 antibody. C, evaluation of IBtkα half-life. HEK293T cells (3 × 10⁶) were treated for 1 h with MG132 (20 μM) or vehicle and then incubated with CHX (100 μg/ml) for up to 16 h. Cell lysates (30 μg) were separated by NuPAGE 4–12% SDS-PAGE and analyzed by WB with the indicated antibodies. **D**, quantification of IBtkα half-life. Protein band intensities of the experiment described in C were normalized to the corresponding GAPDH intensity and then normalized to the 0 h time point (100%). The mean densitometric values ± S.D. (error bars) of three independent experiments are shown.

**TABLE 2**

| Accession no. | Description | Coverage | Unique peptides |
|---------------|-------------|----------|-----------------|
| Q9P2D0        | Inhibitor of Bruton tyrosine kinase | 60.16    | 110             |
| Q86VP6        | Cullin-associated NEDD8-associated protein 1 | 10.81   | 12              |
| P61201        | COP9 signalosome complex subunit 2 | 22.35    | 11              |
| B3KST5        | COP9 signalosome complex subunit 4 | 30.40    | 11              |
| P22314        | Ubiquitin-like modifier-activating enzyme 1 | 8.22    | 11              |
| Q13618        | Cullin3 | 6.99     | 6               |
| F5H265        | Polyubiquitin-C | 63.09    | 22              |
| Q9UNS2-2      | COP9 signalosome complex subunit 3 | 11.66   | 5               |
| Q53E16        | Programmed cell death protein 4 | 12.90   | 3               |

Pdcd4 contains an N-terminal RNA-binding domain and two MA-3 domains located in the central and C-terminal regions (Fig. 4C) (38). To map the amino acid sequence of Pdcd4 required for the binding to IBtkα, wild type and Pdcd4 mutants were used in GST pull-down experiments of cell extracts from IBtkα-FLAG-transfected HEK293T (Fig. 4C). Consistently with in vivo co-IP, IBtkα was pulled down by GST-Pdcd4-WT (Fig. 4D, lane 5). Conversely, IBtkα was slightly pulled down by the Pdcd4-ΔRBD mutant, which lacks the RNA-binding domain (Fig. 4D, lane 6), indicating that the RNA-binding domain of Pdcd4 was required for the binding to IBtkα. Differently, IBtkα was fully recovered by GST-Pdcd4-ΔRBDStop pull-down (Fig. 4D, lane 7), indicating that the MA3 domains of Pdcd4, lacking in the Pdcd4-ΔRBDStop mutant, were dispensable for the binding to IBtkα. By GST pull-down, we also mapped the IBtkα domains required for the binding to Pdcd4, using HEK293 transfected with IBtkα-FLAG mutants. IBtkαAN-FLAG and IBtkαΔBTB-FLAG were recovered by GST-Pdcd4-WT pull-down (Fig. 4E, lanes 7 and 8), indicating that the region encompassing the ankyrin, RCC1, and BTB domains was dispensable for the binding to Pdcd4. Conversely, IBtkαΔC-FLAG did not bind to GST-Pdcd4-WT (Fig. 4E, lane 6), indicating that the C-terminal region of IBtkα, encompassing amino acids 891–1353, included the IBtkα binding site to Pdcd4.

**IBtkα Promotes the Ubiquitylation Coupled to Proteasomal Degradation of Pdcd4**—Next, we tested the hypothesis that IBtkα, as CRL3 component and Pdcd4 interactor, could promote Pdcd4 ubiquitylation coupled to proteasomal degradation. By a ubiquitylation assay in vivo, we observed polyubiqui-
tylated Pdcd4 isoforms in empty vector-transfected HEK293T cells, whose detection was increased by IBtkα-FLAG transfection (Fig. 5A, compare lanes 1 and 2) and reduced by the mutant IBtkαΔBTB-FLAG, which binds to Pdcd4 and not to Cul3 (Fig. 5A, lane 3), supporting the requirement of IBtkα interaction with Cul3 to promote Pdcd4 polyubiquitylation. Further, the overexpression of IBtkα-FLAG also increased the polyubiquitylation of endogenous Pdcd4 (Fig. 5B). Moreover, depletion of IBtkα by RNA interference increased the steady-state Pdcd4 protein level (Fig. 5, C (compare lanes 1 and 3) and D) without significantly affecting the PDCD4 mRNA level (Fig. 5E). The inhibition of protein synthesis with CHX caused a large reduction of Pdcd4 protein level in control shRNA-treated cells (Fig. 5C, lanes 1 and 2), which is consistent with the short half-life of Pdcd4 protein (39). Conversely, Pdcd4 protein level was unaffected in IBtkα depleted and CHX-treated cells (Fig. 5C, lanes 3 and 4), indicating that IBtkα affected the protein amount of Pdcd4 acting at a post-transcriptional level.

Pdcd4 is strictly regulated by serum signaling (39, 40) and undergoes CRL1-mediated ubiquitylation and proteasomal degradation upon serum replenishment in serum-starved cells (39). Thus, we tested whether IBtkα could affect the stability of Pdcd4 in response to serum. To this end, the Pdcd4 protein content was analyzed in CHX time course experiments, where HeLa cells were serum-starved for 16 h and then replenished with serum for the following 5 h, with or without IBtkα RNA interference. Consistent with a previous report (39), Pdcd4 accumulated upon serum starvation as compared with normal serum condition in control siRNA-transfected cells (Fig. 6A, lanes 1 and 2). Conversely, Pdcd4 accumulation was equally observed in IBtkα siRNA-transfected cells with or without serum starvation (Fig. 6A, lanes 3 and 4). When serum was added to starved cells, a slower rate of degradation of Pdcd4 was observed in IBtkα siRNA-transfected cells as compared with untransfected and control siRNA-transfected cells, with a Pdcd4 half-life of 9.2, 2.9, and 3.2 h, respectively (Fig. 6, B and C). These results clearly indicated that IBtkα mediated the Pdcd4 degradation in response to serum because the lack of IBtkα interfered with the serum-induced degradation of Pdcd4. Moreover, the transfection of wild type IBtkα and IBtkαN-FLAG increased the degradation of Pdcd4 as compared with empty vector (Fig. 5, D (lanes 1–6) and E), whereas the mutant IBtkαΔC did not (Figs. 5E and 6D (lanes 7 and 8)). Conversely, the transfection of IBtkαΔBTB-FLAG resulted in a slight
increase of Pdcd4 (Figs. 5E and 6D (lanes 9 and 10)), indicating that IBtk\textsubscript{ΔBTB} behaved as a dominant negative mutant. These results indicated that the amino acid sequences of IBtk\textsubscript{ΔBTB} containing the BTB domains for Cul3 binding and the C terminus for Pdcd4 binding were both required for serum-induced degradation of Pdcd4. Further, MG132 treatment prevented the degradation of Pdcd4 in IBtk\textsubscript{ΔBTB}-transfected cells (Fig. 6F), indicating that IBtk\textsubscript{ΔBTB} promoted the proteasomal degradation of Pdcd4. Finally, Cul3 RNA interference increased the expression of IBtk\textsubscript{ΔBTB}-FLAG significantly increased the degradation of both the Pdcd4-WT-HA and the Pdcd4 S67A/S71A/S76A-HA mutant upon deprivation/replenishment (Fig. 7, A–C). This result indicates that IBtk\textsubscript{ΔBTB}-mediated ubiquitylation of Pdcd4 does not require the presence of serine 67, 71, and 76.

**IBtk\textsubscript{ΔBTB} Modulates the Translational Activity of Pdcd4** — Pdcd4 inhibits the translation of mRNAs with structured 5′-UTR by repressing the eIF4A1 helicase activity (40, 41). In addition, Pdcd4 inhibits the IRES-dependent translation of antiapoptotic proteins, such as Bcl-xL, XIAP (42) and c-Myb (38) through direct binding to the IRES region. Thus, we investigated whether IBtk\textsubscript{ΔBTB} affected the Pdcd4-dependent translation by regulating the Pdcd4 stability. To this end, we performed an in vivo translation assay using two luciferase mRNA reporters containing either a stable stem-loop in the 5′-UTR or an unstructured 5′-UTR (Fig. 8A). By use of this experimental system, Pdcd4 was previously shown to preferentially inhibit the translation of mRNA with stem-loop structured 5′-UTR (41). As compared with mock and siRNA control, depletion of IBtk\textsubscript{ΔBTB} by RNA interference reduced the translation of both mRNA reporters, with a more significant decrease of stem-loop structured 5′-UTR (Fig. 8B). Conversely, depletion of Pdcd4 significantly increased the translation of both mRNA luciferase reporters (Fig. 8B).
As an additional experiment, the transfection of IBtk/H9251-FLAG also increased the translation of both mRNA luciferase reporters, with a greater increase of stem-loop structured 5’-UTR (Fig. 8C). Moreover, IBtk/H9251 and IBtk/H9004C and IBtk/H9251/BTB mutants, lacking the IBtk binding sites for Pdcd4 and Cul3, respectively, did not affect the translation of the luciferase reporters (Fig. 8C). In the context of the luciferase reporter system, these results indicated that IBtkα promoted translation with a preferential effect on mRNA endowed with stem loop structured 5’-UTR, being the IBtkα interaction domains with Pdcd4 and Cul3 required for this action.

We also determined whether IBtkα affected the global protein synthesis and the translation of Bcl-XL as a Pdcd4-specific mRNA target (42). In HeLa cells, IBtkα depletion by RNA interference did not affect the IBtkα-dependent Pdcd4 degradation (Fig. 8D); however, it significantly decreased the Bcl-XL protein content while increasing Pdcd4 (Fig. 8E). As control, the levels of Bcl-XL and Pdcd4 transcripts were unaffected by IBtkα RNA interference (Fig. 8F). These results indicated that IBtkα enhanced the translation of a Pdcd4-dependent transcript, such as Bcl-XL, by affecting the Pdcd4 stability.

**Discussion**

BTB proteins can regulate several cellular processes by promoting the recruitment of degradation targets to E3 ubiquitin ligase complexes. In this study, we have demonstrated that...
IBtkα, an uncharacterized BTB protein, is substrate receptor of a Cul3-dependent ubiquitin ligase, here named CRL3<sup>IBTK</sup>. In fact, we have demonstrated the in vivo association of IBtkα with Cul3 by co-immunoprecipitation of endogenous or ectopically expressed proteins in HEK293T cells, which is consistent with previous reports on physical interaction of IBtkα with Cul3 in 293T cells (43), mouse embryonic stem cells (44, 45), and NKT lymphocytes (44). Then we have shown that the BTB domains of IBtkα and the N terminus of Cul3 mediated the association of the two proteins, which is consistent with the requirement of the BTB domain for binding to the N terminus of Cul3 (11, 13). In addition, we showed that the 3-box motif at the C-terminal side of the IBtkα BTB2 domain stabilized the binding of IBtkα to Cul3, which was consistent with the 3-box motif being a structural feature of most BTB proteins of the CRL3 complex (12). Indeed, Btkα assembled within a classical CRL3 complex in vivo, which included Cul3 and Rbx1, and underwent Lys<sup>48</sup> polyubiquitylation. This behavior was similar to that of other substrate receptors, representing a characteristic signature of cellular E3 feedback regulation mediated by autoubiquitylation (36).

Due to the occurrence of ankyrins, RCC1, and BTB domain within the same polypeptide (26), IBtkα is endowed with unique biochemical and functional properties as compared with other CRL3 substrate adaptors, because it could share multiple physical and functional interactions with members of cellular pathways. In the search of CRL3<sup>IBTK</sup> substrates, we found that IBtkα in vivo associated with Pdcd4, a tumor suppressor involved in several cellular processes, including transcription repression (37). Indeed, we proved that IBtkα promoted the ubiquitylation and subsequent proteosomal degradation of Pdcd4. In fact, the depletion of IBtkα by RNA interference caused the Pdcd4 accumulation without altering the PDCD4 gene expression. Conversely, overexpression of IBtkα promoted the Pdcd4 degradation, and this effect was counteracted by the proteasome inhibitor MG132, indicating that the Pdcd4 ubiquitylation mediated by CRL3<sup>IBTK</sup> was coupled to proteosomal degradation. Moreover, the mutant IBtkαΔBTB, which bound to Pdcd4 and not to Cul3, was unable to promote Pdcd4 ubiquitylation, suggesting the requirement of IBtkα interaction with Cul3 to promote the Pdcd4 ubiquitylation. Collectively, our results indicated that CRL3<sup>IBTK</sup> targeted Pdcd4 for ubiquitylation coupled to proteosomal degradation.

Dorrello et al. (39) previously showed that in starved T98G glioblastoma cells, Pdcd4 was ubiquitylated/degraded following serum starvation/replenishment through the SCF<sup>BTrcp</sup>-dependent pathway, which required the phosphorylation of Pdcd4 at serines 67, 71, and 76. In the present study, we have found that CRL3<sup>IBTK</sup> also promotes the Pdcd4 ubiquitylation/degradation upon serum starvation/replenishment. However, the IBtkα-dependent degradation of Pdcd4 does not require the presence of serines 67, 71, and 76 because the Pdcd4 S67A/S71A/S76A mutant, which is resistant to the SCF<sup>BTrcp</sup>-mediated degradation (39), still underwent to IBtkα-dependent degradation. These results indicate that the IBtkα-mediated degradation of Pdcd4 relies on regulatory mechanisms that differ from the SCF<sup>BTrcp</sup> pathway. In this regard, proteins may be...
subjected to redundant ubiquitylation through multiple E3 ligases. For example, cyclin E1 is ubiquitylated by CRL3RhoBTB3 (46) or SCFFbx7 during entry into S phase of the cell cycle (47). Thus, it is possible that different E3s cooperate in regulating the turnover of ligase targets whenever specific stimuli generate the appropriate signals to trigger the rapid ubiquitylation and degradation of substrates. Having identified CRL3IBTK as adaptor of Pdcd4 for CRL3-dependent ubiquitylation, it will be relevant in the future to characterize the specific pathways activating this process.

Pdcd4 inhibits the translation of mRNAs with a structured 5'-UTR by repressing the eIF4A1 RNA helicase (40) as well as the assembly of distinct mRNAs, such as Bcl-xL, with the indicated antibodies. F. HeLa cells were transfected as described in E, and total RNA was analyzed by real-time PCR to measure the level of the indicated transcripts. Mean values ± S.D. of three independent experiments are shown.

By using two luciferase mRNA reporters, we observed that depletion of IBtkα by RNA interference inhibited the translation of mRNAs with stem-loop structured or unstructured 5'-UTR. HeLa cells (3 × 10^6) were transfected with IBtk siRNA, Pdcd4 siRNA, or control siRNA or left untransfected (mock). After 24 h, cells were transfected with pCMV-LUC (0.2 μg) or pCMV-SL-LUC (0.2 μg) and serum-starved for 12 h, followed by growth in complete medium (10% FBS) for additional 24 h. The luciferase activity measured in untransfected cells was designated as 100%. Mean values ± S.D. (error bars) of five independent experiments are shown. C, overexpression of IBtkα enhances the translation of reporter mRNAs with stem-loop structured or unstructured 5'-UTR. HeLa cells (3 × 10^6) were transfected with IBtkα-FLAG, IBtkα-FLAG mutants, or empty vector (4 μg). Subsequent steps were performed as described in B. D, IBtkα RNA interference does not affect the global protein synthesis. HeLa cells (3 × 10^6) were transfected with IBtk siRNA or control siRNA or left untransfected (mock), and 48 h later, cell lysates were analyzed by WB with the indicated antibodies. F. HeLa cells were transfected as described in E, and total RNA was analyzed by real-time PCR to measure the level of the indicated transcripts. Mean values ± S.D. of three independent experiments are shown.

FIGURE 8. IBtkα enhances translation by counteracting the Pdcd4 repression of target mRNAs. A, schematic representation of luciferase reporter mRNAs. B, depletion of IBtkα by RNA interference decreases the translation of reporter mRNAs with stem-loop structured or unstructured 5'-UTR. HeLa cells (3 × 10^6) were transfected with IBtk siRNA, Pdcd4 siRNA, or control siRNA or left untransfected (mock). After 24 h, cells were transfected with pCMV-LUC (0.2 μg) or pCMV-SL-LUC (0.2 μg) and serum-starved for 12 h, followed by growth in complete medium (10% FBS) for additional 24 h. The luciferase activity measured in untransfected cells was designated as 100%. Mean values ± S.D. (error bars) of five independent experiments are shown. C, overexpression of IBtkα enhances the translation of reporter mRNAs with stem-loop structured or unstructured 5'-UTR. HeLa cells (3 × 10^6) were transfected with IBtkα-FLAG, IBtkα-FLAG mutants, or empty vector (4 μg). Subsequent steps were performed as described in B. D, IBtkα RNA interference does not affect the global protein synthesis. HeLa cells (3 × 10^6) were transfected with IBtk siRNA or control siRNA or left untransfected (mock), and 48 h later, cell lysates were analyzed by WB with the indicated antibodies. F. HeLa cells were transfected as described in E, and total RNA was analyzed by real-time PCR to measure the level of the indicated transcripts. Mean values ± S.D. of three independent experiments are shown.
dependent pathways (50). Obtaining further insights into the regulation mechanisms of the CRL3_{IBTK} activity will probably clarify the specific role of IBtkα in different signaling pathways.

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