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βTrCP- and Rsk1/2-Mediated Degradation of BimEL Inhibits Apoptosis

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SUMMARY

The BimEL tumor suppressor is a potent proapoptotic BH3-only protein. We found that, in response to survival signals, BimEL was rapidly phosphorylated on three serine residues in a conserved degron, facilitating binding and degradation via the F box protein βTrCP. Phosphorylation of the BimEL degron was executed by Rsk1/2 and promoted by the Erk1/2-mediated phosphorylation of BimEL on Ser69. Compared to wild-type BimEL, a BimEL phosphorylation mutant unable to bind βTrCP was stabilized and consequently potent at inducing apoptosis by the intrinsic mitochondrial pathway. Moreover, although non-small cell lung cancer (NSCLC) cells often become resistant to gefitinib (a clinically relevant tyrosine kinase inhibitor that induces apoptosis through BimEL), silencing of either *βTrCP* or *Rsk1/2* resulted in BimEL-mediated apoptosis of both gefitinib-sensitive and gefitinib-insensitive NSCLC cells. Our findings reveal that βTrCP promotes cell survival in cooperation with the ERK-RSK pathway by targeting BimEL for degradation.

INTRODUCTION

Bim (Bcl-2 Interacting Mediator of cell death) is a powerful, proapoptotic member of the Bcl-2 protein family expressed mainly in hematopoietic, epithelial, neuronal, and germ cells (O'Reilly et al., 2000). Alternative mRNA splicing generates three major isoforms: short (BimS), long (BimL), and extra long (BimEL), with BimEL the predominant isoform in most tissues (O'Connor et al., 1998; O'Reilly et al., 2000). Bim plays a key role in linking stress-induced signals to the intrinsic (mitochondrial) apoptotic pathway. Upon exposure to stress, such as growth factor deprivation, Bim activates proapoptotic Bak and Bax that, in turn, permeabilize the mitochondrial membrane, causing the release of cytochrome *c* and the consequent activation of caspases to cause programmed cell death. Mechanistically, Bim is thought to activate Bax and Bak by direct binding and/or by binding and inhibiting antiapoptotic members of the Bcl2 family (Mcl1 and Bcl-XL), which restrain Bak and Bax (Fletcher and Huang, 2008).

Several studies suggest that Bim functions as a tumor suppressor. In mice, inactivation of one allele of *Bim* accelerates Myc-induced B cell leukemia (Egle et al., 2004). In human cancers, Bim is eliminated via various mechanisms to provide a growth advantage to the tumor cells. Homozygous deletions of the *Bim* locus have been reported in mantle cell lymphomas, and methylation of the *Bim* promoter is found in certain Burkitt's lymphomas and diffuse large B cell lymphomas (Mestre-Escorihuela et al., 2007; Tagawa et al., 2005). Moreover, in a manner similar to other tumor suppressor proteins, such as p27 and p53, Bim levels are decreased in transformed cells via enhanced protein degradation, particularly when the ERK pathway is constitutively activated. For example, in transformed epithelial cells (both in culture and in animals), paclitaxel-induced apoptosis is mediated by Bim (Tan et al., 2005). When the H-Ras/ERK pathway is activated in tumor cells, BimEL is eliminated by proteasomal degradation, and cells become refractory to paclitaxel. Treatment with bortezomib, a proteasome inhibitor, restores BimEL levels, thereby resensitizing cells to paclitaxel.

Bim levels are also low in non-small cell lung cancer (NSCLC) cells harboring activating EGFR mutations (Costa et al., 2007; Cragg et al., 2007; Deng et al., 2007a; Gong et al., 2007). Inhibition of EGFR tyrosine kinase activity using drugs such as gefitinib results in BimEL accumulation and, consequently, induction of apoptosis. Similarly, BimEL accumulation mediates imatinib-induced cell death of Bcr/Abl+ leukemic cells (Belloc et al., 2007; Kuroda et al., 2006).

The proteasomal degradation of BimEL is promoted by phosphorylation by Erk1/2 on a specific serine (Ser69 in human) (Hubner et al., 2008; Ley et al., 2003, 2004, 2005; Luciano et al., 2003). However, despite the importance of BimEL in determining cell fate and the fact that its degradation enables tumor cells to escape chemotherapy-induced apoptosis, the cellular machinery responsible for BimEL degradation has not yet been

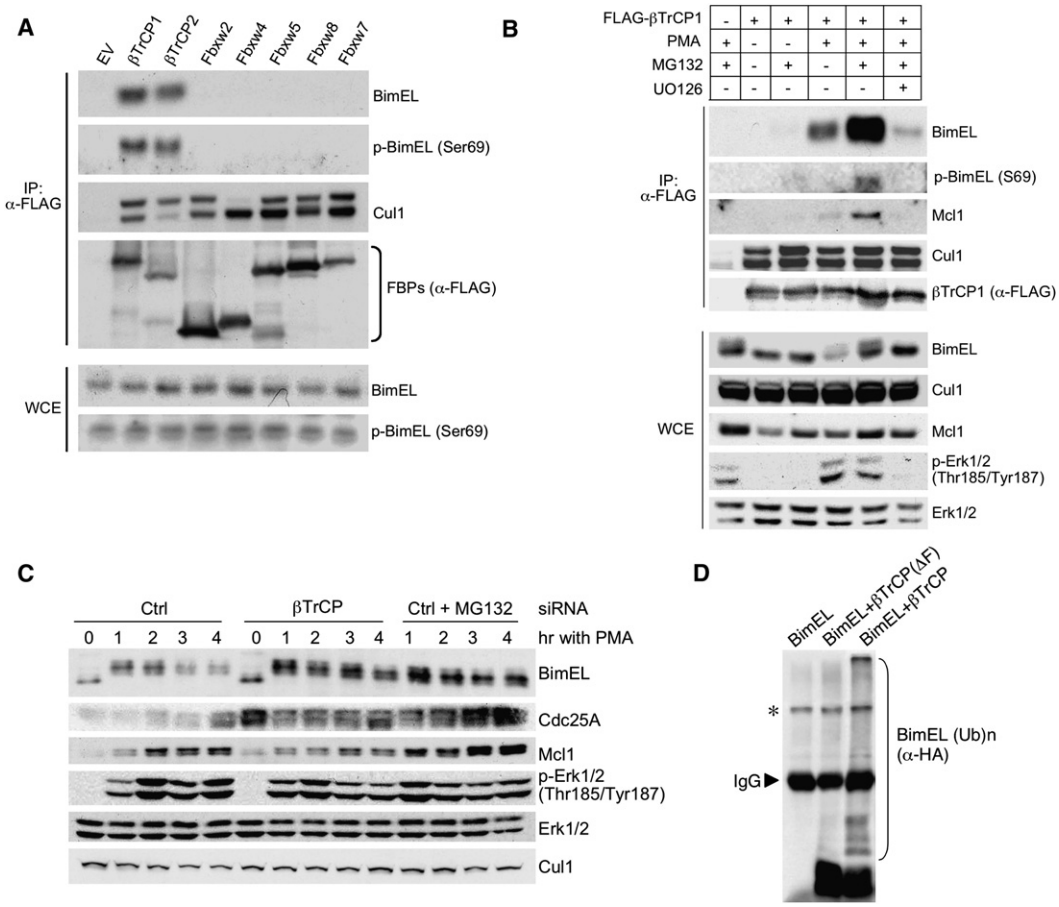

Figure 1. βTrCP Controls the Degradation of BimEL

(A) BimEL specifically interacts with βTrCP. HEK293 cells were transfected with the indicated FLAG-tagged F box proteins (FBPs) or an empty vector (EV). Twenty-four hours posttransfection, cells were treated for 3 hr with PMA and MG132 before harvesting. Whole-cell extracts (WCE) were subjected to immunoprecipitation (IP) with anti-FLAG resin (α-FLAG) and immunoblotting for the indicated proteins.

(B) The interaction of βTrCP1 with BimEL is promoted by PMA and MG132 and is inhibited by a MEK inhibitor. HEK293 cells were transfected with FLAG-tagged βTrCP or an empty vector. Twenty-four hours posttransfection, cells were treated with the indicated drugs for 3 hr before harvesting. Whole-cell extracts (WCE) were subjected to immunoprecipitation (IP) with anti-FLAG resin (α-FLAG) and immunoblotting for the indicated proteins.

(C) β*TrCP* silencing stabilizes BimEL. HEK293 cells were treated with a control siRNA (Ctrl) or an siRNA targeting β*TrCP*. Forty-eight hours posttransfection, cells were treated with either PMA alone or PMA and MG132 for the indicated times. Extracts were then immunoblotted for the indicated proteins. The accumulation of Cdc25A (a known βTrCP substrate) demonstrates efficient β*TrCP* knockdown (also confirmed by RT-PCR [Figure S10]).

(D) HEK293 cells were transfected with HA-tagged BimEL, Skp1, Cul1, and Roc1 in the presence of an empty vector (EV), FLAG-tagged βTrCP, or FLAG-tagged βTrCP(ΔF box). After immunopurification with anti-FLAG resin, in vitro ubiquitylation of BimEL was performed. Samples were analyzed by immunoblotting with an anti-HA antibody. The asterisk denotes a nonspecific band.

identified. The study described herein identifies the ubiquitin ligase and kinases that target BimEL for proteasomal degradation, elucidating a critical control mechanism for the apoptotic response.

RESULTS

Degradation of BimEL is promoted by ERK-dependent phosphorylation on Ser69. As phosphorylation often targets proteins to SCF (Skp1-Cullin1-F-box protein) ubiquitin ligase complexes, we asked whether BimEL binds to Cul1 in HEK293 cells. We found that in the presence of PMA, an activator of the ERK pathway, Cul1, but not Cul2, was able to coimmunoprecipitate endogenous BimEL (see Figure S1 available online). We then investigated which F box protein specifically targets BimEL to the SCF. Screening of the FBXW (F box, WD repeat) family proteins revealed that endogenous BimEL specifically interacts with βTrCP1 and βTrCP2 (Figure 1A), two paralogous F box proteins that (to date) share identical biochemical properties and substrates (Frescas and Pagano, 2008). (The term βTrCP will refer to both, unless specified.) The interaction of endogenous BimEL and βTrCP1 was also observed (Figure S2). Addition of PMA to HEK293 cells promoted the binding of endogenous BimEL to βTrCP1, and binding was enhanced when the proteasome inhibitor MG132 blocked BimEL degradation (Figure 1B). However, the binding of BimEL to βTrCP1 in the presence of

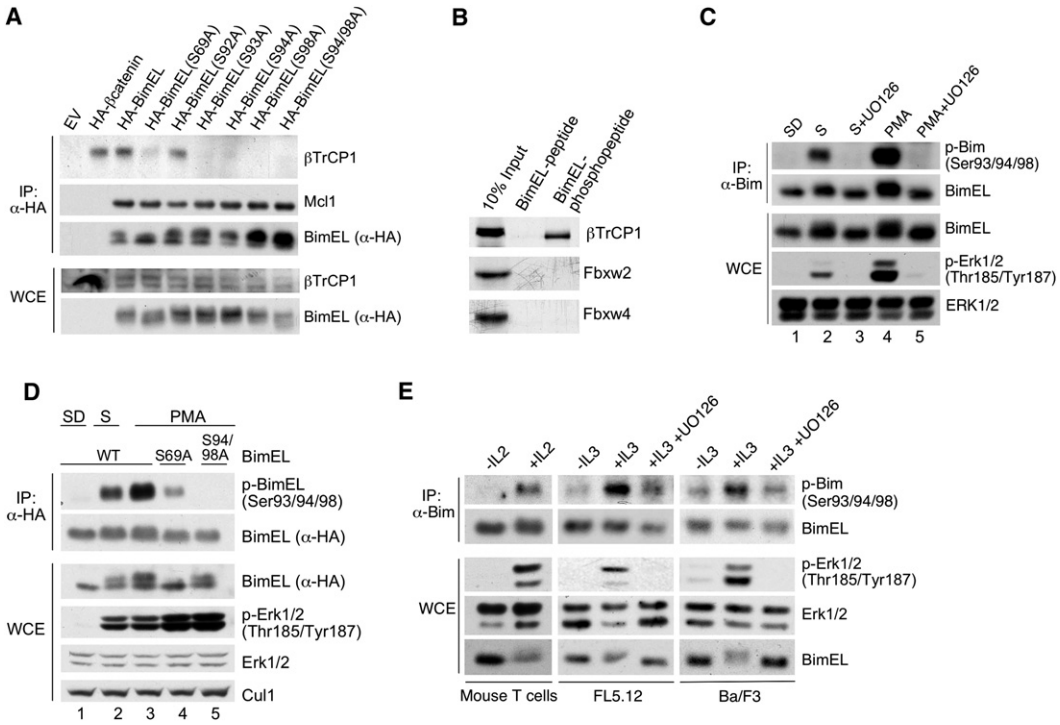

Figure 2. Identification of the BimEL Degron

(A) Ser93, Ser94, and Ser98 are required for the interaction of BimEL with βTrCP1. HEK293 cells were transfected with an empty vector (EV), HA-tagged β-catenin (positive control), HA-tagged wild-type BimEL, or the indicated HA-tagged BimEL mutants. Twenty-four hours posttransfection, cells were treated for 3 hr with PMA and MG132. Whole-cell extracts (WCE) were subjected to immunoprecipitation (IP) with anti-HA resin (α-HA) and immunoblotting for the indicated proteins.

(B) The BimEL degron requires phosphorylation to bind βTrCP1. ^{35}S-labeled, in vitro-translated βTrCP1, Fbxw2, and Fbxw4 were used in binding reactions with beads coupled to the BimEL peptide ^{88}CLSRSSSGYFSFD^{100} (lane 2) or the phosphopeptide ^{88}CLSRSpSpSGYFpSFD^{100} (lane 3). Beads were washed with lysis buffer, and bound proteins were eluted and subjected to SDS-PAGE and autoradiography. The first lane shows 10% of the in vitro-translated protein inputs.

(C) In vivo phosphorylation of BimEL on Ser93/94/98 is induced by mitogens. HEK293 cells were serum deprived (SD) for 24 hr (lane 1), and then either serum (S) (lanes 2 and 3) or PMA (lanes 4 and 5) was added for 20 min in the absence or presence of UO126, as indicated. Whole-cell extracts (WCE) were subjected to immunoprecipitation (IP) with an anti-Bim antibody and immunoblotting for the indicated proteins.

(D) Ser69 promotes phoshorylation of BimEL on Ser93/94/98. HEK293 cells were transfected with HA-tagged wild-type (WT) BimEL (lanes 1–3), HA-tagged BimEL(S69A) (lane 4), or HA-tagged BimEL(S94/98A) (lane 5). Cells were serum deprived (SD) for 24 hr (lane 1), and then either serum (S) (lane 2) or PMA (lanes 3–5) was added for 20 min. Whole-cell extracts (WCE) were subjected to immunoprecipitation (IP) with anti-HA resin (α-HA) and immunoblotting for the indicated proteins.

(E) Cytokines induce phosphorylation of BimEL on Ser93/94/98. Activated primary mouse T cells were deprived of IL2 for 5 hr, and Ba/F3 and FL5.12 cells were deprived of IL3 for 5 hr. Then cells were stimulated for 15 min with IL2 or IL3, in the absence or presence of UO126, as indicated. Whole-cell extracts (WCE) were subjected to immunoprecipitation with an anti-Bim antibody and immunoblotting for the indicated proteins.

PMA and MG132 was strongly reduced when PMA-induced ERK activation was inhibited with UO126, a MEK inhibitor.

These results indicated that the binding of BimEL to βTrCP is stimulated by the activation of Erk1/2, suggesting (consistent with previous evidence that Erk1/2 induce BimEL degradation) that βTrCP controls the phosphorylation-dependent degradation of BimEL. We therefore investigated this hypothesis by reducing the expression of both βTrCP1 and βTrCP2 in HEK293 cells using a previously validated siRNA. Figure 1C shows that βTrCP silencing counteracted the effect of PMA on BimEL degradation, stabilizing BimEL. Finally, immunopurified βTrCP1, but not an inactive βTrCP1(ΔF box) mutant, induced the in vitro ubiquitylation of BimEL (Figure 1D), supporting the hypothesis that the effect of βTrCP on BimEL is direct.

βTrCP binds its substrates via phosphorylated residues in a conserved degron, typically the consensus sequence DpSGXXpS. In searching for a βTrCP degron in human BimEL, we found the conserved motif ^{91}RSSSGYFSFD^{100} (Figure S3A), in which the charge on the aspartic acid is potentially substituted by phosphorylated Ser93. This sequence fits into the three-dimensional structural space of the βTrCP1 substrate-binding surface, similar to the phosphodegron of β-catenin, a known substrate of βTrCP (Figures S3C–S3E). To test whether BimEL binds βTrCP via this motif, we generated a number of serine to alanine mutants (Figure S3B) and tested their binding to endogenous βTrCP1. Single mutations of Ser93, Ser94, and Ser98 to Ala or a double Ser94/98Ala mutation (S94/98A) abrogated the interaction between BimEL and endogenous βTrCP1, although they did not abolish BimEL binding to endogenous Mcl1 (Figure 2A). Mutation of Ser92 did not inhibit BimEL binding to either βTrCP1 or Mcl1.

To investigate whether phosphorylation plays a role in the interaction with βTrCP, we used immobilized, synthetic peptides

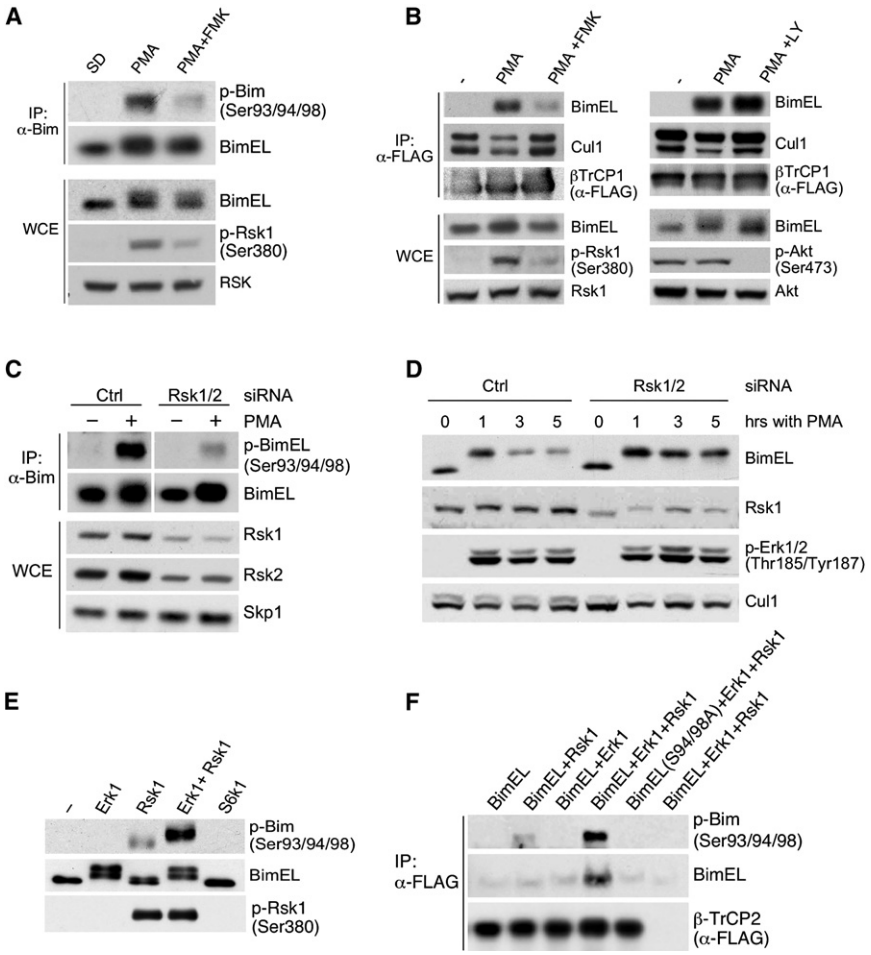

Figure 3. Rsk1/2 Control BimEL Stability

(A) Inhibition of Rsk1/2 reduces phosphorylation of BimEL on Ser93/94/98. HEK293 were serum deprived (SD) and then treated for 15 min with PMA in the absence or presence of FMK, as indicated. Whole-cell extracts (WCE) were subjected to immunoprecipitation with an anti-Bim antibody and immunoblotting for the indicated proteins.

(B) Inhibition of Rsk1/2 reduces BimEL binding to βTrCP1. HEK293 cells were transfected with FLAG-tagged βTrCP1. Twenty-four hours post-transfection, cells were treated with PMA and, where indicated, either FMK or LY294002 (LY) was added. Whole-cell extracts (WCE) were subjected to immunoprecipitation (IP) using FLAG resin (α-FLAG) and immunoblotting for the indicated proteins.

(C) HEK293 cells were treated with a control siRNA (Ctrl) or siRNAs targeting both *Rsk1* and *Rsk2* mRNAs. Cells were deprived of serum for 24 hr (−) and then activated with PMA (+) for 20 min. Cell extracts were subjected to immunoprecipitation with an anti-Bim antibody and immunoblotting for the indicated proteins.

(D) Silencing *Rsk1/2* stabilizes BimEL. HEK293 cells were treated with a control siRNA (Ctrl) or siRNAs targeting both *Rsk1* and *Rsk2*. Forty-eight hours after siRNA treatment, cells were treated with PMA for the indicated times. Extracts were immunoblotted for the indicated proteins.

(E) Rsk1 phosphorylates BimEL in vitro. Recombinant, purified BimEL was incubated for 30 min with ATP and the indicated purified kinases. Reaction products were subjected to immunoblotting for the indicated proteins.

(F) In vitro binding of BimEL to βTrCP2 is dependent on Rsk1. Recombinant, purified wild-type BimEL and BimEL(S94/98A) were phosphorylated as in (E) and incubated with in vitro-translated, FLAG-tagged βTrCP2 (except the sample shown in the last lane). Proteins were immunoprecipitated (IP) using FLAG resin (α-FLAG), and immunoblotting for the indicated proteins was performed.

spanning the candidate phosphodegron (aa 88–100). While a peptide containing phosphorylated Ser93, Ser94, and Ser98 efficiently bound βTrCP1 (but not FBXW2 and FBXW4), a corresponding, nonphosphorylated peptide was unable to bind βTrCP1 (Figure 2B), in agreement with the idea that phosphorylation of Ser93, Ser94, and Ser98 directly mediates the interaction with βTrCP. Furthermore, only in vitro phosphorylated BimEL binds βTrCP2 (see later, Figure 3F).

To further investigate the role of BimEL phosphorylation, we generated a phospho-specific antibody against the ^{88}CLSRSpSpSGYFpSFD^{100} peptide with phosphoserines at positions 93, 94, and 98. This antibody recognized wild-type BimEL, but not a BimEL(S93/94/98A) mutant, while single and double amino acid BimEL mutants displayed decreasing levels of detection (Figure S4A), indicating that all three serine residues are phosphorylated and contribute to recognition by this antibody. In addition, λ-phosphatase treatment of immunopurified BimEL abolished BimEL recognition by the phospho-specific antibody (Figure S4B). Using this reagent, we tested BimEL phosphorylation under different conditions in vivo. We found that both endogenous and exogenous BimEL were rapidly phosphorylated in HEK293 cells in response to mitogenic stimulation (serum or PMA), which correlated with ERK activation (Figures 2C and 2D). In contrast, BimEL was not phosphorylated in serum-starved cells or UO126-treated cells. Similarly, cytokines also promoted phosphorylation of BimEL on its degron, as shown with IL2 in primary mouse T cells or IL3 in FL5.12 and BaF/3 cell lines (Figure 2E).

Interestingly, mutation of Ser69 to Ala strongly inhibited phosphorylation of the BimEL degron despite PMA treatment (Figure 2D). Accordingly, the BimEL(S69A) mutant bound less efficiently to endogenous βTrCP1 (Figure 2A). These results indicate that phosphorylation on Ser69 promotes the phosphorylation of Ser93, Ser94, and Ser98 (see also later, Figure 3E and Figure S7A). Such a bipartite mechanism has been previously demonstrated for other substrates of βTrCP that require combinatorial phosphorylation by two cooperative kinases (Hunter, 2007).

Erk1/2-mediated phosphorylation of Ser69 is well established. Thus, we pursued the identification of the kinase that phosphorylates the BimEL degron. GPS (Group-Based Prediction System), a kinase prediction program (Xue et al., 2008), detected

consensus sites for Rsk1/2 and S6k1/2 in the BimEL degron. To gain insight into the kinase involved in BimEL phosphorylation, we used pharmacological inhibitors and found that FMK (an RSK inhibitor [Cohen et al., 2005]), but not LY294002 (a PI3K inhibitor, which, consequently, inhibits also S6k1/2), strongly reduced both the phosphorylation of BimEL on Ser93/Ser94/Ser98 and the binding of BimEL to βTrCP1 (Figures 3A and 3B). Similarly, another RSK inhibitor, BI-D1870 (Sapkota et al., 2007), reduced BimEL-βTrCP1 interaction too (data not shown). We also found that Rsk1, but not S6k1, was coimmunoprecipitated with BimEL in vivo (Figure S5). All these results suggest that Rsk1 and Rsk2 phosphorylate the BimEL degron. Accordingly, knockdown of both Rsk1 and Rsk2 with two validated siRNAs inhibited the PMA-induced phosphorylation of Ser93/Ser94/Ser98 in both endogenous and exogenous BimEL (Figure 3C and Figure S6A). Importantly, downregulation of Rsk1/2 inhibited BimEL degradation (Figure 3D), and BI-D1870 treatment induced BimEL accumulation (Figure S6B).

To test whether Rsk1 can directly phosphorylate the BimEL degron, we performed an in vitro kinase assay using recombinant, bacterially expressed, purified BimEL and kinases. Rsk1 phosphorylated the degron of BimEL, as shown by the appearance of a slow-migrating band and recognition by our phospho-specific antibody, and this event was promoted by Erk1 (Figure 3E). In contrast, neither Erk1 nor S6k1 alone was able to induce phosphorylation of BimEL on Ser93/Ser94/Ser98. Addition of Erk1 did not increase the activating phosphorylation of Rsk1 (bottom panel of Figure 3E), and when Erk1 was first used to phosphorylate BimEL (and washed away prior to Rsk1 addition), the stimulation by ERK was observed for wild-type BimEL, but not for BimEL(S69A) (Figure S7A). Thus, the enhancement of the Rsk1-dependent phosphorylation of BimEL is not due to the activation of Rsk1 by Erk1; instead, it is promoted by the phosphorylation of BimEL on Ser69 by Erk1. We also used single serine mutants and found that Rsk1 was able to phosphorylate (in a ERK-dependent manner) each of the three serines in the BimEL degron (Figures S7B–S7D), suggesting that, like other established substrates (Anjum and Blenis, 2008), Rsk1 and Rsk2 target multiple residues in BimEL. Finally, in agreement with the phosphorylation results, the in vitro binding of βTrCP2 to phosphorylated BimEL was dependent on Rsk1 and stimulated by Erk1 (Figure 3F).

The above data strongly support a model in which phosphorylation of BimEL on Ser93/Ser94/Ser98 mediates binding to βTrCP and degradation via $SCF^{\beta TrCP}$. Therefore, failure to bind βTrCP should result in stabilization of BimEL. To test this hypothesis, we transfected wild-type BimEL or BimEL(S94/98A) into HEK293 cells and subsequently treated with PMA and cycloheximide. As predicted, in contrast to wild-type BimEL, BimEL(S94/98A), which does not bind βTrCP (Figure 2A), was not degraded upon PMA treatment (Figure 4A). Importantly, expression of BimEL(S94/98A) in immortalized $Bim^{-/-}$ mouse embryo fibroblasts (MEFs) triggered a much more robust apoptotic response than that obtained by expressing wild-type BimEL or even BimEL(S69A) (Figure 4B). Neither wild-type BimEL nor BimEL mutants induced apoptosis in immortalized $Bak^{-/-}$;$Bax^{-/-}$ MEFs, confirming that BimEL(S94/98A)-dependent cell death occurs via the intrinsic mitochondrial pathway.

We also asked whether RSK and βTrCP mediate survival of primary human $CD4^+$ T cells. Figure 4C shows that the silencing of either Rsk1/2 or βTrCP in these cells resulted in BimEL accumulation and BimEL-mediated apoptosis (as demonstrated by the return of cell death to background levels when BimEL was downregulated together with Rsk1/2 or βTrCP). Accordingly, primary mouse T cells from wild-type mice, but not from $Bim^{-/-}$ mice, died in response to pharmacologic inhibition of RSK (Figure S8).

To further study the biological significance of the βTrCP- and Rsk1/2-mediated degradation of BimEL, we used NSCLC cells that harbor activating mutations in the epidermal growth factor receptor (EGFR). Initially, clinically relevant inhibitors of EGFR tyrosine kinase activity, such as gefitinib, trigger a BimEL-dependent apoptotic response in NSCLCs with EGFR mutations (Costa et al., 2007; Cragg et al., 2007; Deng et al., 2007b; Gong et al., 2007). However, these tumors eventually become resistant to tyrosine kinase inhibitors and lose their ability to die via BimEL upregulation. We examined two EGFR mutant NSCLC cell lines, HCC827 (which are known to be sensitive to gefitinib) and H1650 (which are not) (see also Figure 4E). Significantly, in the absence of gefitinib, apoptosis was promoted in both HCC827 and H1650 cells when either Rsk1/2 or βTrCP was downregulated (Figures 4D–4F). When BimEL was also silenced, cell death returned to background levels (Figures 4E and 4F). Notably, upregulation of BimEL correlated with the induction of apoptosis in H1650 and HCC827 cells. These experiments showed that restoration of BimEL levels in cells harboring activating mutations in EGFR promotes apoptosis in both gefitinib-sensitive and gefitinib-insensitive NSCLC cells.

DISCUSSION

Despite the importance of BimEL in controlling apoptotic responses, the ubiquitin ligase responsible for its degradation had remained elusive. Two ligases, c-Cbl and a Cul2 complex, have been proposed to target BimEL (Akiyama et al., 2003; Zhang et al., 2008), but these findings have not been confirmed by others (El Chami et al., 2005; Wiggins et al., 2007; Figure S1). Indeed, we show here that, in response to survival signals, $SCF^{\beta TrCP}$ promotes the degradation of BimEL in cooperation with Erk1/2 and Rsk1/2.

Degradation of BimEL enables tumor cells to escape chemotherapy-induced apoptosis. We found that silencing of either βTrCP or Rsk1/2 induces Bim-dependent apoptosis in NSCLC cells harboring activating mutations in EGFR, irrespective of their sensitivity to gefitinib. In a clinical setting, such an increase in cell death could positively affect long-term outcomes, so our findings suggest that inhibition of RSK or βTrCP should be pursued as a rational and valid therapeutic strategy to induce apoptosis of tumor cells in NSCLC and, possibly, other malignancies. Furthermore, since BimEL degradation is dependent on Rsk1/2, but only stimulated by Erk1/2, it is expected that tumor cells that undergo BimEL-dependent death (e.g., in NSCLCs, Bcr/Abl+ leukemias, and certain breast cancers) may be more sensitive to RSK inhibitors than to ERK inhibitors.

In summary, we describe the biochemical and molecular details of the mechanisms controlling the degradation of BimEL

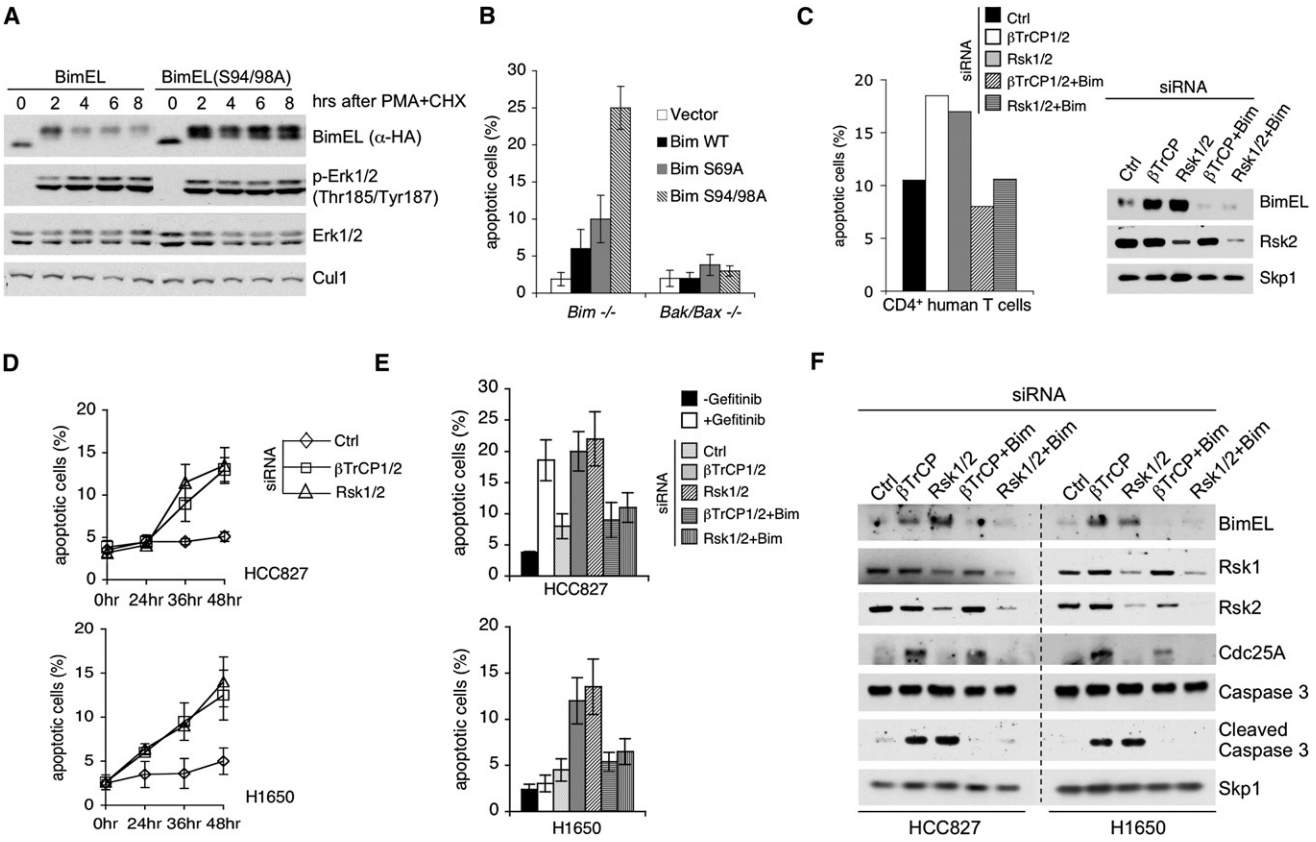

Figure 4. SCF$^{\beta TrCP}$- and Rsk-Mediated Degradation of BimEL Controls the Apoptotic Response

(A) Mutation of Ser94/98 stabilizes BimEL despite ERK activation. Cells were transfected with either wild-type BimEL or BimEL(S94/98A) mutant. Twenty-four hours posttransfection, cells were treated with PMA and cyclohexamide (CHX) for the indicated times before immunoblotting for the indicated proteins.

(B) Mutation of Ser94/98 augments the apoptotic activity of BimEL. *Bim*$^{-/-}$ and *Bax*$^{-/-}$;*Bak*$^{-/-}$ MEFs were infected with a retrovirus expressing either wild-type BimEL or different BimEL mutants. Apoptosis was measured 48 hr following infection using propidium iodide and Annexin V staining, with flowcytometric analysis (n = 3, ±SD).

(C) Silencing *Rsk1/2* or *βTrCP* promotes Bim-dependent apoptosis in primary human T cells. Human T cells were transfected twice with the indicated siRNAs and collected 24 hr thereafter. Apoptosis (left panel) was determined as in (B), and cell extracts were analyzed by immunoblotting for the indicated proteins (right panel).

(D) Silencing *Rsk1/2* or *βTrCP* promotes apoptosis in NSCLC cells independent of their sensitivity to gefitinib. HCC827 and H1650 cells were transfected with the indicated siRNAs and collected at the indicated times. Apoptosis was determined as in (B) (n = 3, ±SD).

(E) Silencing *Bim* rescues apoptosis induced by downregualtion of *Rsk1/2* or *βTrCP*. HCC827 and H1650 cells were treated with gefitinib for 24 hr or transfected with the indicated siRNAs and collected 48 hr thereafter. Apoptosis was determined as in (B) (n = 3, ±SD).

(F) HCC827 and H1650 cells, treated as in (E), were collected, and cell extracts were analyzed by immunoblotting for the indicated proteins.

in both normal and cancer cells. When cells are stimulated with mitogens, βTrCP directs the degradation of BimEL in cooperation with the ERK-RSK pathway, resulting in the inhibition of cell death. At the same time, in cooperation with the PI3K-S6K pathway, βTrCP targets Pdcd4 for degradation, allowing efficient protein synthesis and, consequently, cell growth (Dorrello et al., 2006). Thus, βTrCP coordinates cell survival and cell growth in response to mitogenic stimuli (Figure S9).

EXPERIMENTAL PROCEDURES

Biochemical Methods

Extract preparation, immunoprecipitation, and immunoblotting were previously described (Dorrello et al., 2006). In vitro ubiquitylation was previously described (Busino et al., 2007).

Antibodies

Mouse monoclonal antibodies were from Invitrogen (Cul1, Erk1/2, Rsk1, Rsk2, βTrCP1), Sigma (anti-FLAG), Santa Cruz Biotechnology (Cdc25A), BD Biosciences (β-catenin), and Covance (anti-HA). Rabbit polyclonal antibodies were from Invitrogen (Cul1, Bim, Skp2), Biosource (phospho-Bim[Ser69], phospho-Erk1/2[Thr185/Tyr187]), Cell Signaling (phosho-RSK[Ser380], Bim, caspase-3, cleaved caspase-3), Bethyl Laboratories (βTrCP1), and Santa Cruz Biotechnology (Mcl1). For immunoprecipitation of endogenous Bim, we used a rat monoclonal antibody from Millipore. The phospho-specific antibody to BimEL was generated using the phosphopeptide CLSRSpSpSGYFpSFD.

Plasmids

BimEL mutants were generated using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Both wild-type BimEL and BimEL mutants were subcloned into the pBabe retroviral vector. All cDNAs were completely sequenced.

Transient Transfections and Retrovirus-Mediated Gene Transfer

Transfections using the calcium phosphate and retrovirus-mediated gene transfer were previously described (Dorrello et al., 2006).

Apoptosis Assay

Apoptosis was assessed using Annexin V-FITC and propidium iodide staining (BD Pharmigen).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and ten figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(08)00890-3.

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