Title
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Proto-oncogene CT10-regulated kinase (CRK) is a pro-apoptotic transducer of endoplasmic reticulum stress

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Abstract

Excessive demands on the protein folding capacity of the endoplasmic reticulum (ER) cause irremediable ER stress and contribute to cell loss in a number of cell degenerative diseases, including type 2 diabetes and neurodegeneration[1,2]. The signals communicating catastrophic ER damage to the mitochondrial apoptotic machinery remain poorly understood[3-6]. We used a biochemical approach to purify a cytosolic activity induced by ER stress that causes release of cytochrome *c* from isolated mitochondria. We discovered that the principal component of the purified pro-apoptotic activity is proto-oncogene CT10-regulated kinase (CRK), an adaptor protein with no known catalytic activity[7]. *Crk*$^{-/-}$ cells are strongly resistant to ER stress-induced apoptosis. Moreover, CRK is cleaved in response to ER stress to generate an N-terminal ~14kD fragment with greatly enhanced cytotoxic potential. We identified a putative BCL2 homology-3 (BH3) domain within this N-terminal CRK fragment, which sensitizes isolated mitochondria to cytochrome *c* release and when mutated significantly reduces CRK's apoptotic activity *in vivo*. Together these results identify CRK as a pro-apoptotic protein that signals irremediable ER stress to the mitochondrial execution machinery.

Irremediable ER stress represents a form of intrinsic cell damage that culminates in activation of the BAX/BAK-dependent mitochondrial apoptotic pathway[3,4]. Homo-oligomerization of BAX and/or BAK consequently results in outer mitochondrial membrane permeabilization and release of pro-death mitochondrial proteins (e.g., cytochrome *c*) into the cytosol, causing activation of effector caspases[8-10]. For many forms of cell injury, including ER stress, we have a limited understanding of the cellular transducers that relay the information of upstream damage to BAX/BAK oligomerization at mitochondria. To

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AUTHOR CONTRIBUTIONS

K.A. and E.T.J. designed and performed experiments and contributed to the manuscript. T-J.P and T.C. contributed reagents and data interpretation. S.A.O. designed the study and wrote the manuscript.

COMPETING INTERESTS STATEMENT

None to report.

date, the only known BAX and/or BAK activators are members of the pro-apoptotic BH3-only family, a diverse class of polypeptides containing a loosely conserved ~9-12 amino acid BH3 death domain[11,12,13]. The BH3-only proteins BID, BIM, NOXA and PUMA have been previously implicated in ER stress-induced death[14,15]; however, cells deficient in one or more of these proteins are not completely resistant to this form of apoptosis[16-18]. Therefore, it is highly probable that additional proteins that communicate ER stress to the mitochondrial apoptotic machinery remain to be discovered. To pursue this possibility, we took an unbiased biochemical approach to purify the major ER stress-induced cytosolic pro-apoptotic activity.

Given their strong resistance to ER stress-induced apoptosis [16,17], we reasoned the *Bax*$^{-/-}$*Bak*$^{-/-}$ mouse embryonic fibroblasts (MEFs) present a unique and powerful tool to trap and identify pre-mitochondrial apoptotic signals activated by ER stress. Therefore, we challenged SV40-transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs with the pharmacological agent Brefeldin A (BFA), which blocks ER-golgi protein transport, to induce irremediable ER stress and initiate the pre-mitochondrial apoptotic program. We prepared cytosolic extracts (S100) from untreated or BFA-treated *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs, then incubated isolated Jurkat mitochondria with these extracts, and measured the amount of mitochondrial cytochrome *c* released as a readout for pro-apoptotic activity. The S100 of BFA-treated *Bax*$^{-/-}$*Bak*$^{-/-}$ cells triggers the release of ~90% of total intra-mitochondrial cytochrome *c* in a BAX/BAK-dependent manner[15], while the S100 fraction from untreated *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs induces negligible (<5%) cytochrome *c* release (Fig. 1a). Thus, ER stress induces a cytosolic activity capable of releasing mitochondrial cytochrome *c*, which we have termed ***C***ytochrome ***c*** ***R***eleasing ***A***ctivity (CcRA). We previously found that proteolytically active BID (tBID) is responsible for a portion of this CcRA (~30%), but that the majority of CcRA remains intact in BFA-treated *Bid*$^{-/-}$ S100[15]. Therefore, we designed and performed a biochemical purification strategy to isolate additional CcRA factors in the BFA-treated *Bax*$^{-/-}$*Bak*$^{-/-}$ S100 (Fig. 1b).

The CcRA-containing fractions from the final step (MonoQ gradient) (Fig. 1c) of the purification scheme did not contain detectable amounts of any known BH3-only protein by immunoblotting (data not shown), and so were analyzed by MALDI mass spectrometry (Fig. 1c). Proto-oncogene CT10-regulated kinase (CRK) was the highest confidence protein identified by mass spectrometry in the active fractions, with approximately 25% of the total sequence represented in 6 tryptic peptides (Supplementary Table 1). *C-crk* encodes two splice isoforms, CRKI (28kD) and CRKII (38kD)[7], which have been previously recognized as adaptor components in multi-protein complexes involved in cell morphology, movement, proliferation, and differentiation[19]. CRKI and CRKII share a common Src homology 2 (SH2) domain and SH3 domain, while CRKII contains an additional C-terminal SH3 domain (Fig. 1d)[19]. As the peptides detected by mass spectrometry are common to both CRKI and CRKII, this information did not differentiate which isoform is present in the analyzed fractions (Supplementary Table 1).

To test if CRK plays a role in ER stress-induced apoptosis in cells, we challenged *crk*$^{-/-}$ and wild-type (WT) matched MEFs with BFA or tunicamycin (TUN), an ER stress agent that specifically inhibits *N*-linked glycosylation. As previously reported, these *crk*$^{-/-}$ MEFs are

derived from genetically engineered embryos that fail to express either CRKI or CRKII[20] (Fig. 2a). Notably, S100 from BFA-treated *crk*$^{-/-}$ MEFs has significantly decreased CcRA compared to that from BFA-treated WT MEFs (Fig. 2b), indicating that CRK is required for the majority of the ER stress-induced apoptotic signal. In addition, *crk*$^{-/-}$ MEFs are strikingly resistant to ER stress-induced apoptosis, but as sensitive as WT MEFs to staurosporine (STS), a pan-kinase inhibitor known to activate the mitochondrial apoptotic pathway independently of ER stress, thereby confirming the pro-apoptotic role of CRK specifically in the ER stress-induced apoptotic pathway, rather than generally from other intrinsic stresses (Fig. 2c, d).

To investigate which CRK isoform is necessary for ER stress-induced apoptosis, we reconstituted *crk*$^{-/-}$ MEFs by transient transfection with expression constructs encoding either *crkI* or *crkII* and evaluated their sensitivity to ER stress-induced apoptosis. Surprisingly, *crkI* and *crkII* are both able to independently rescue the sensitivity of *crk*$^{-/-}$ MEFs to ER stress-induced apoptosis, arguing that a sequence common to CRKI and CRKII is required for CRK's apoptotic activity (Fig. 3a,b). We next attempted to use retroviral vectors to establish stable cells lines of *crk*$^{-/-}$ MEFs expressing either *crkI* or *crkII*. While we had no difficulty recovering cells reconstituted with *crkII*, all attempts to establish stable *crkI* expression in *crk*$^{-/-}$ MEFs were unsuccessful, indicating that CRKI may be inherently cytotoxic. Stable reconstitution of *crkII* in *crk*$^{-/-}$ MEFs restores sensitivity to ER stress agents, but does not affect the response to STS (Fig. 3c-e), recapitulating the transient expression results (Fig. 3a). Moreover, stable overexpression of *crkII* and transient overexpression of *crkI* further sensitizes WT MEFs to ER stress (Fig. 3f-i). Together, these data strongly argue that CRK is a critical component of the ER stress-induced apoptotic pathway.

To explore its potential role in ER stress, we monitored CRK mRNA and protein following BFA treatment (Supplementary Fig. S1). We detected no changes in CRKI or CRKII mRNA transcript levels upon ER stress (Supplementary Fig. S1). However, we found that ER stress causes depletion of full-length CRKII (Supplementary Fig. S2). To determine if CRKII is reduced in a specific subcellular compartment, we probed subcellular fractions from *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs and found that CRKII is partially localized to the ER, mitochondria, and cytosol (Fig. 4a). Moreover, upon ER stress, 38kD ER- and cytosol- localized CRKII is reduced, while levels of the mitochondrion-localized full-length CRKII change very little (Fig. 4a). Interestingly, we found that upon ER stress, CRKII appears to be sequentially cleaved at least twice, resulting in several distinct fragments (Fig. 4a and Supplementary Fig. S3). Proteolytic cleavage is a post-translational modification recognized to activate other known pro-apoptotic proteins, such as BID, but had not been described previously for CRK[21-24].

We tested if CRKI also undergoes cleavage events upon ER stress. Following ER stress, at least one CRKI-specific cleavage product is readily observed (Fig. 4b). In addition, we observe depletion of full-length endogenous CRKI and CRKII in both WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs upon BFA treatment (Fig. 4c), indicating that their cleavage occurs upstream of the mitochondrial apoptotic pathway. Furthermore, the loss of full-length CRKI and CRKII

occurs only when the level of ER stress rises to cytotoxic levels, and correlates with the initiation of ER stress-induced apoptosis (Supplementary Fig. S4)[25-28].

In an attempt to determine the role of CRK cleavage for its apoptotic activity, we tested a small panel of protease inhibitors for their ability to block this event (data not shown). We found that the pan-cysteine protease inhibitor ZVAD-FMK prevents ER stress-induced loss of full-length CRKI and CRKII and protects *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs from the cytopathic effects of ER stress (Supplementary Fig. S5). We previously identified caspase-2 as a pre-mitochondrial cysteine protease activated by ER stress[10]. However, caspase-2 is poorly inhibited by ZVAD-FMK and *in vitro* experiments with recombinant caspase-2 do not result in CRK cleavage (data not shown). These data suggest that a previously unidentified ER stress-activated cysteine protease is responsible for CRK cleavage.

To determine if the observed ER stress-induced cleavage of CRK is critical for its apoptotic activity, we individually mutated each potential cysteine protease cleavage site (aspartic acid) in the CRK sequence. When stably reconstituted into *crk*$^{-/-}$ MEFs, CRKII ΔD110A (*crk*$^{-/-}$ *+crkII (ΔD110A)*) was the only aspartic acid mutant unable to be cleaved in response to ER stress (Fig. 4d). Furthermore, non-cleavable CRKII ΔD110A is defective in restoring *crk*$^{-/-}$ MEF sensitivity to ER stress-induced apoptosis (Fig. 4e, f), arguing that this cleavage event is critical for its apoptotic activity. Cleavage at D110 is predicted to produce one fragment of approximately 25kD, which can be detected by a C-terminal-specific antibody (Fig 4d), and a second N-terminal fragment of ~14kD, which is undetectable using available antibodies. As both CRKI and CRKII restore *crk*$^{-/-}$ sensitivity to ER stress, it is likely the shared N-terminal fragment (~14kd) contains the critical domain for its apoptotic function. To test this prediction, we transiently expressed the N-terminally FLAG-tagged fragment (NF110) in the absence of ER stress (Fig. 4g) and measured apoptosis. As predicted, NF110 is able to potently induce cell death independently of ER stress (Fig. 4h, i). From these data, we conclude that CRK is cleaved upon ER stress at D110, to produce a pro-apoptotic fragment. Further studies will be necessary to identify the upstream protease and its connection to the unfolded protein response pathway.

To further investigate the mechanism by which CRK triggers cell death, we tested if CRK-induced apoptosis is a BAX/BAK-dependent process. Transient overexpression of CRKI or CRKII triggers apoptosis in WT MEFs, but not *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs, confirming that both isoforms signal upstream of the BAX/BAK-dependent mitochondrial apoptotic pathway (Fig. 5a-d). Pro-apoptotic BH3-only proteins are the only known molecules capable of activating BAX and/or BAK either directly or by inhibiting anti-apoptotic BCL-2 family proteins[4,13]. Through sequence analysis, we identified a putative BH3-like domain within CRK that contains a number of conserved amino acids present in several recognized BH3-only proteins (Fig. 5e). This sequence is present in both *crk* splice forms and located within the common N-terminal 110 a.a. pro-apoptotic fragment (Fig. 1d, 5e). In support of our hypothesis that CRK contains a BH3-like domain, we determined that CRK is capable of binding a prototypical anti-apoptotic BCL-2 family protein (BCL-XL), a common feature of most BH3-only proteins. Following ER stress induction, transiently expressed FLAG-CRKII co-immunoprecipitates with BCL-XL on FLAG-specific agarose beads (Fig. 5f).

To determine if this sequence has BH3-like pro-apoptotic activity, we treated isolated Jurkat mitochondria with a synthetic CRK BH3 domain (77-96 a.a.) and measured cytochrome *c* release. There are two classes of BH3-only domains, those that sensitize or activate BAX/BAK-dependent mitochondrial apoptosis[29]. BH3 domains that directly "activate" BAX and/or BAK at mitochondria, such as the BH3 domain of BID, are able to cause cytochrome *c* release from isolated mitochondria. In contrast, "sensitizing" BH3 domains, such as the BH3 domains of BAD and BIK, sensitize isolated mitochondria to release cytochrome *c* in the presence of a second "activating" BH3 domain. *In vivo*, sensitizer BH3-only proteins are thought to competitively bind anti-apoptotic proteins, releasing bound "activating" BH3-only proteins to induce mitochondrial permeability. While the CRK BH3 domain is unable to induce cytochrome *c* release alone, it significantly potentiates with low concentrations of truncated BID (tBID) to cause cytochrome *c* release (Fig. 5g).

To further examine if this putative BH3 domain is required for CRK's pro-apoptotic activity in cells, we mutated the highly conserved aspartic acid (*D91A*) in *crkII* and evaluated the ability of this mutant to rescue the *crk*$^{-/-}$ MEF phenotype. In comparison to wild-type *crk*, the BH3 mutant *crk (D91A)* has significantly decreased apoptotic activity, arguing that this region is critical for its pro-death signaling (Fig. 5h, i).

Together, these results identify CRK as a major pro-apoptotic signal required for the execution of ER stress-induced cell death. During ER stress, CRKI and CRKII are cleaved by a yet to be identified cysteine protease to generate an N-terminal fragment with potent apoptotic activity. Furthermore, CRK interacts with anti-apoptotic BCL-XL and its apoptotic activity is upstream of the BAX/BAK-dependent mitochondrial pathway. Both CRK isoforms contain a putative BH3 domain, which sensitizes isolated mitochondria to tBID-induced cytochrome *c* release and when mutated diminishes apoptotic activity in cells. These data argue that CRK is a previously unidentified BH3-only-like protein, which upon ER stress is proteolytically processed into a pro-death signal. Our findings suggest that CRK may be a valuable therapeutic target in diseases where ER stress-induced cell loss is implicated, including some forms of neurodegeneration and diabetes[1,2].

We have identified a previously unknown pro-apoptotic function common to both CRK isoforms. CRK was initially identified through its homology with transforming *v-crk*[7,30,31]. However, only CRKI has been shown to have transforming activity in some cell culture models, and it is upregulated in a number of human cancers[32,33]. Clues that CRK mediates apoptosis are present in other species. For example, the *C. elegans* Crk-homologue CED-2 regulates apoptotic engulfment[34,35]. Mammalian CRKII has been reported to induce death in some transformed cell types upon overexpression[36,37] and is required for apoptotic activity that can be detected in *Xenopus* egg extracts[38-40]. Our work is the first to connect CRK to apoptosis, specifically under ER stress, in mammalian cells.

In addition to the shared pro-apoptotic function we have discovered for CRKI and CRKII, there are notable and possibly functionally significant differences between the isoforms. Our inability to establish a cell line stably overexpressing CRKI, in contrast to multiple cell lines stably overexpressing CRKII, suggests that CRKI may be the more cytotoxic isoform. Indeed, the expression of endogenous CRKI is restricted to approximately 10% that of

CRKII (Supplementary Fig. S1), perhaps to limit its toxicity. In support of this notion, we observe that CRKI is cleaved more efficiently and at earlier kinetics than CRKII in response to ER stress (Fig. 4c and Supplementary Fig. S4). These observations and our discovery that CRKI can be converted into a pro-apoptotic protein in response to ER stress raise the possibility that pharmacologic inducers of ER stress may have therapeutic efficacy in cancers where *crkI* is upregulated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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MATERIALS AND METHODS

Cellular Fractionation and Cytochrome c Release Assay

MEFS were resuspended in mitochondria isolation buffer (200mM sucrose, 10mM Tris/MOPS (morpholinepropanesulfonic acid) [pH 7.4], 1mM EGTA) plus 1x protease inhibitor cocktail (PIC, Sigma) and manually disrupted by shearing the suspension 10x through a 27-gauge and then 10x through a 30-gauge needle. Mitochondria (heavy membrane) were isolated from the suspension with an initial 700×g 4°C centrifugation to remove nuclei, followed by a 7000×g 4°C centrifugation to isolate heavy membrane (mitochondrial) fraction. The endoplasmic reticulum (light membrane) was isolated from the cytosolic fraction (S100) by a 100,000×g 4°C centrifugation. Mitochondrial and ER fractions were resuspended in RIPA (150mM NaCl, 1% NP40, 0.5% DOC, 0.1% SDS, 50mM Tris [pH 8.0]). S100 was dialyzed for 2 hours into 10% glycerol-containing mitochondria experimental buffer (MEB; 125mM KCl, 10mM Tris/MOPS [pH 7.4], 5mM glutamate, 1.25mM malate, 2μM EGTA, 1μM KPhos). Jurkat mitochondria for the cytochrome *c* releasing assay were isolated as described above, minus the second shearing through the 30-gauge needle. For the cytochrome *c* releasing assay reaction, 100-200μg (sample dependent) of S100 extract was incubated with 50μg of isolated Jurkat mitochondria for 45 minutes at room temperature. Following the incubation, the supernatant and pellet were separated by a 4°C 16,000×g centrifugation. The remaining cytochrome *c* was released from mitochondria by resuspending the pellet in PBS plus 0.05% Triton-X. The amount of cytochrome *c* present in the supernatant and lysed pellets was quantified using a human cytochrome *c* linked immunosorbent assay (ELISA)(R&D systems) per the manufacturer's instructions. The percentage of cytochrome *c* release was calculated by dividing the amount of cytochrome *c* present in the supernatant by the sum of cytochrome *c* present in both the supernatant and pellet fractions.

Purification of Cytochrome c Releasing Activity

The ER stress apoptotic factor was purified using the CcRA assay described above utilizing an AKTA FPLC (GE Healthcare). Briefly, 804mg of S100 protein isolated from 24h BFA (2.5 μg/ml) treated *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs was initially precipitated with 40-80% saturated ammonium sulfate. This active fraction was then further purified on a Phenyl Sepharose column (GE Healthcare) by loading in 1M Ammonium Sulfate and eluting with a 100mM Na_2HPO_4 – 100mM Na_2HPO_4 gradient. The CcRA eluted in the 100.925-62.875ms/cm range and was further purified on a MonoP column (GE Healthcare) gradient pH 6-10. The CcRA eluted between pH 9-6. This fraction was then purified further on the Superdex 200 column (GE Healthcare) and eluted in the 10-44kD range. This CcRA containing-fraction was run over a MonoQ column (GE Healthcare) 10mM KCl-1M KCl gradient. The purified CcRA fraction was analyzed in-gel and in-solution by MS-MS mass spectrometry by the UCSF Biomolecule Core Facility and the Stanford University of Medicine, Protein and Nucleic Acid Facility (PAN).

Cell culture and biological reagents

SV40 transformed *Bax*$^{-/-}$*Bak*$^{-/-}$ and WT control MEFs were passaged as previously described[17]. *Crk*$^{-/-}$ and WT control MEFs were 3T3 immortalized. All MEFs were maintained in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin, 2mM glutamine, and nonessential amino acids (UCSF cell culture facility). Human Jurkat cells were maintained in RPMI supplemented with 10% fetal bovine serum, 100U/ml penicillin, 100ug/ml streptomycin, 2mM glutamine, 100mM HEPES, and nonessential amino acids (UCSF cell culture facility). Annexin-V FITC was purchased from BioVision. Protease inhibitor cocktail (PIC), IPTG (isopropyl-4-D-thiogalactopyranoside), thapsigargin (TG), brefeldin A (BFA), staurosporine (STS), tunicamycin (TUN), and ZVAD-FMK were purchased from Sigma.

Antibodies and Western Blot analysis

Antibodies used for Western Blot analysis include anti-CRKII (Sigma)(1:1000), anti-CRK (BD Biosciences)(1:1000), anti-Mn SOD (assay designs)(1:500), anti-SERCA (affinity bioreagents)(1:1000), anti-BCL-XL (santa cruz technologies)(1:500), anti-FLAG M2 (Sigma)(1:1000) and anti-ACTIN (Sigma)(1:1000). Secondary antibodies were purchased from Jackson ImmunoResearch (anti mouse, anti-rat, and anti-rabbit antibodies) and Bio-Rad (anti-goat antibody)(1:2000).

Transient transfection and stable cell line selection

MEFs were transiently transfected using Lipofectamine 2000 (Invitrogen) per the manufacturer's instructions. 293 cells were transfected using Fugene6 (Promega) per the manufacturer's instructions. The *crkI* and *crkII* stable cell lines were made by transfecting a retroviral packaging line (293GPG or Phoenix) with *pmx-crkI*, *pmx-crkII*, and *plpcx-crkII* plasmids. Virus was harvested and titered from the supernatants of these packaging cell

lines. Following infection with the virus, stable lines were isolated using 1.25μg/ml puromycin selection.

Plasmid Construction

C-terminal FLAG-crkII was cloned using the following primers: *5*'-TTTGGATCCCGCCACCATGGCGGGCAACTTCGACTCGGAGGAG, 3'-AAAGCGGCCGCTCACTTGTCATCGTCGTCCTTGTAGTCGCTGAAGTCCTCATCGGG. *Crk* aspartic acid mutants were generated using Stratagene Quickchange Lightning Site-directed Mutagenesis Kit and the following primers; D39A (5'-GGTGTTCCTGGTGCGGGCCTCGAGCACCAGCCCCG, 3'-CGGGGCTGGTGCTCGAGGCCCGCACCAGGAACACC), D46A (5'-GAGCACCAGCCCCGGGGCCTATGTGCTTAGCG, 3'-CGCTAAGCACATAGGCCCCGGGGCTGGTGCTC), D110A (5'-CACTATTTGGCCACTACAACATTGATAGAACCAGTGGCC, 3'-GGCCACTGGTTCTATCAATGTTGTAGTGGCCAAATAGTG), D142A (5'-TGTCGGGCCCTCTTTGCCTTTATAGGGAATGATG, 3'-TCATTCCCATTAAAGGCAAAGAGGGCCCGCACA), D150A (5'-GGGAATGATGAAGAAGCTCTTCCCTTTAAGAAAGGAGAC, 3'-GTCTCCTTTCTTAAAGGGAAGAGCTTCTTCATCATTCCC), D157A (5'-CCCTTTAAGAAAGGAGCCATCCTGAGAATCCGGG, 3'-CCCGGATTCTCAGGATGGCTCCTTTCTTAAAGGG), D163A (5'-CCTGAGAATCCGGGCTAAGCCTGAAGAGCAGTGGTGG, 3'-CCACCACTGCTCTTCAGGCTTAGCCCGGATTCTCAGG), D174A (5'-GTGGTGGAATGCAGAGGCCAGCGAAGGAAAGAGGG, 3'-CCCTCTTTCCTTCGCTGGCCTCTGCATTCCACCAC), D252A (5'-GCGAGTCCCTAATGCCTACGCCAAGACAGCCTTGGC, 3'-GCCAAGGCTGTCTTGGCGTAGGCATTAGGGACTCGC). N-terminal FLAG-tagged *crk* (110 a.a.) was cloned using 5'-CGGCCAAGCTTCGCCACCATGGACTACAAGGACGA and 3'-GGCCGGCGGCCGCTCAGTCCAAATAGTGTATTTTGTAG. *Crk* BH3 mutant was generated using Stratagene Quickchange Lightning Site-directed Mutagenesis Kit and these primers; 5'-CCCTCCAGGCTCCGAATAGCAGATCAAGAATTTGA, 3'-TCAAATTCTTGATCTGCTATTCGGAGCCTGGAGGG.

qPCR

Crk isoform mRNA levels were quantitated using qpCR. Sybrgreen (applied biosystems) was used to measure *crk* isoforms with primers designed to recognize only *crkII* (primer sequence: 5'-ATGGCGGGCAACTTCGACT, 3'-CATCGGGATTCTGTTGATCC). The amount of *crkII* mRNA was subtracted from total *crk* mRNA (primer sequence: 3'-CCCTCCTGGTTACCTCCAAT) in order to quantitate the amount of *crkI* mRNA present.

Annexin V staining and FACS analysis

Cells were harvested with 0.25% trypsin, washed once with 1x PBS, and incubated with Annexin V binding buffer (2 mM CaCl2, 80 mM NaCl, 1% HEPES plus 1μg/ml fluorescein isothiocyanate-annexin V) for 5 min. The cells were subsequently passed through a FACSCalibur machine (Becton Dickinson) and detected with CellQuest software (BD Biosciences). Statistical analyses were completed using Student's *t* test.

Immunoprecipitation

Approximately 2.5×10^4 293 cells were transiently transfected using Fugene (Roche) per manufacturer's protocol for 24h with 2μg of plasmid encoding *crkII* wild-type or N-terminal Flag-tagged *crkII*. These cells were then treated with 1.25μg/ml BFA for 12h. Cells were then trypsinized, lysed in RIPA (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% Sodium Deoxycholate, 1% Triton X 100), and 200μg of whole cell lysate was incubated for 2h with anti-FLAG M2 affinity gel beads (SIGMA). The FLAG affinity beads were pelleted by centrifugation at 1200rpm for 5 minutes. The reaction supernatant was removed and concentrated using microcon centrifugal filter devices (3,000 MWCO)(amicon). The beads were washed four times in 1x phosphate buffered saline. SDS-containing loading dye was added to both the concentrated supernatant and the beads. Samples were boiled for 8 minutes and run on a 10% Bis-Tris gel (Invitrogen). The transferred gel was immunoblotted for endogenous BCL-XL using anti-BCL-XL (santa cruz technologies)(1:500) and anti-FLAG M2 (Sigma)(1:1000).

CRK synthetic peptide

The CRK BH3 domain peptide was synthesized by ELIM biopharm. Sequence synthesized: QPPPGVSPSRLRIGAQEFDS.

BH3 mitochondria sensitization assay

Jurkat mitochondria were isolated as described above and resuspended in MEB. The indicated dilutions of recombinant truncated BID (tBID) and 100μM CRK BH3 domain peptide were incubated with 50μg of isolated Jurkat mitochondria for 45 minutes at room temperature. Following the incubation, the supernatant and pellet were separated by a 4°C 16,000×*g* centrifugation. The remaining cytochrome *c* was released from mitochondria by resuspending the pellet in PBS plus 0.05% Triton-X. The amount of cytochrome *c* present in the supernatant and lysed pellets was quantified using a human cytochrome *c* linked immunosorbent assay (ELISA)(R&D systems) per the manufacturer's instructions. The percentage of cytochrome *c* release was calculated by dividing the amount of cytochrome *c* present in the supernatant by the sum of cytochrome *c* present in both the supernatant and pellet fractions.

Statistical Analysis

Unpaired two-tailed Student's *t*-test was used to analyze flow cytometric and ELISA sample data.

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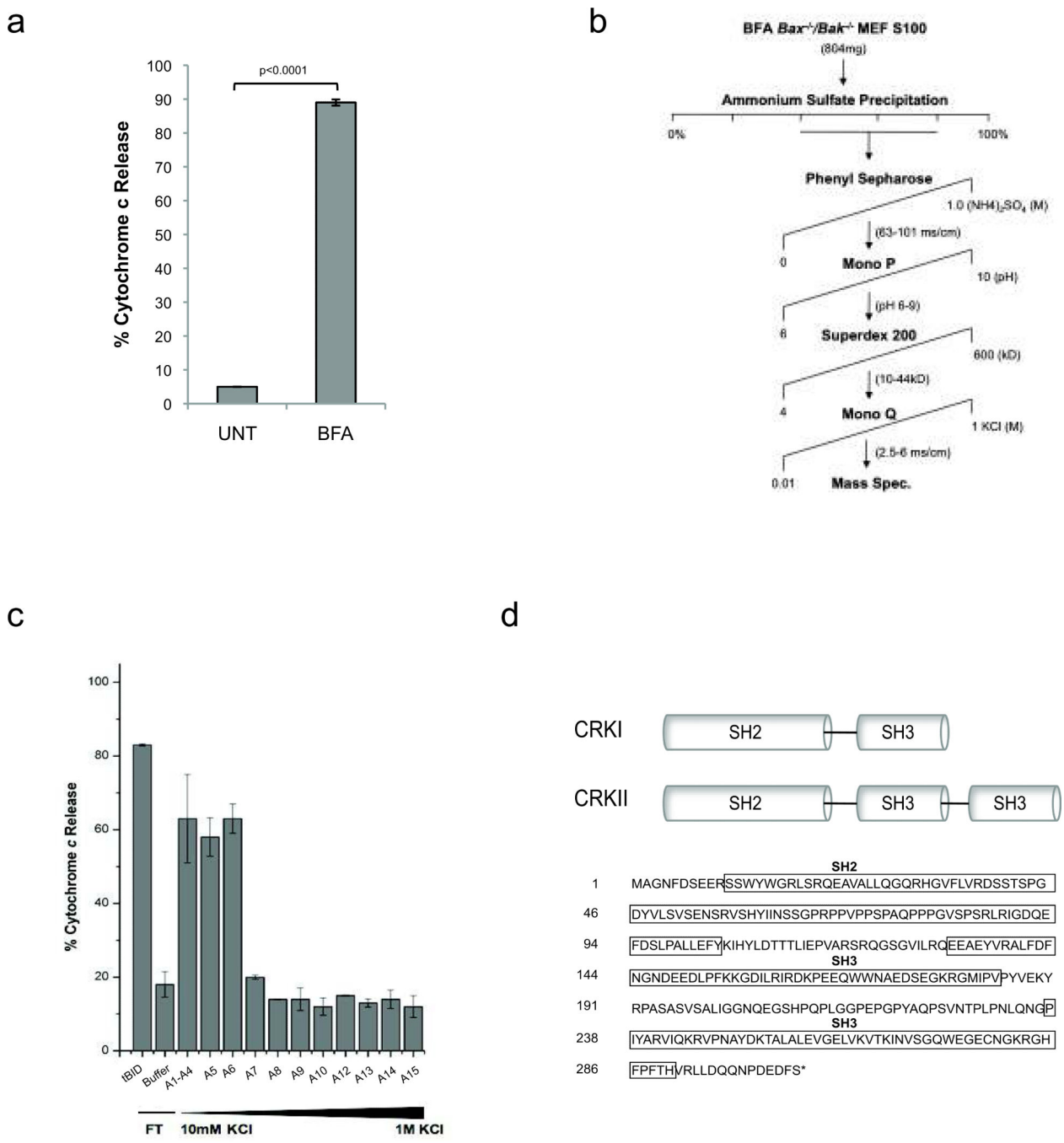

Figure 1. Biochemical purification of ER stress apoptotic activity identifies proto-oncogene CT-10-regulated kinase (CRK)

(**a**) Induction of cytochrome *c* release from isolated Jurkat mitochondria by cytosolic extracts (S100) from untreated (UNT) and 24h Brefeldin A (BFA) 2.5 μg/ml treated *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. (**b**) FPLC purification scheme of cytochrome *c* releasing activity (C*c*RA) present in BFA *Bax*$^{-/-}$*Bak*$^{-/-}$ S100. Active fractions from each purification step are indicated. (**c**) CcRA assay of the fractions from the final step of the purification (MonoQ ion exchange gradient). (**d**) Diagram of CRK isoforms, domains, and amino acid sequence.

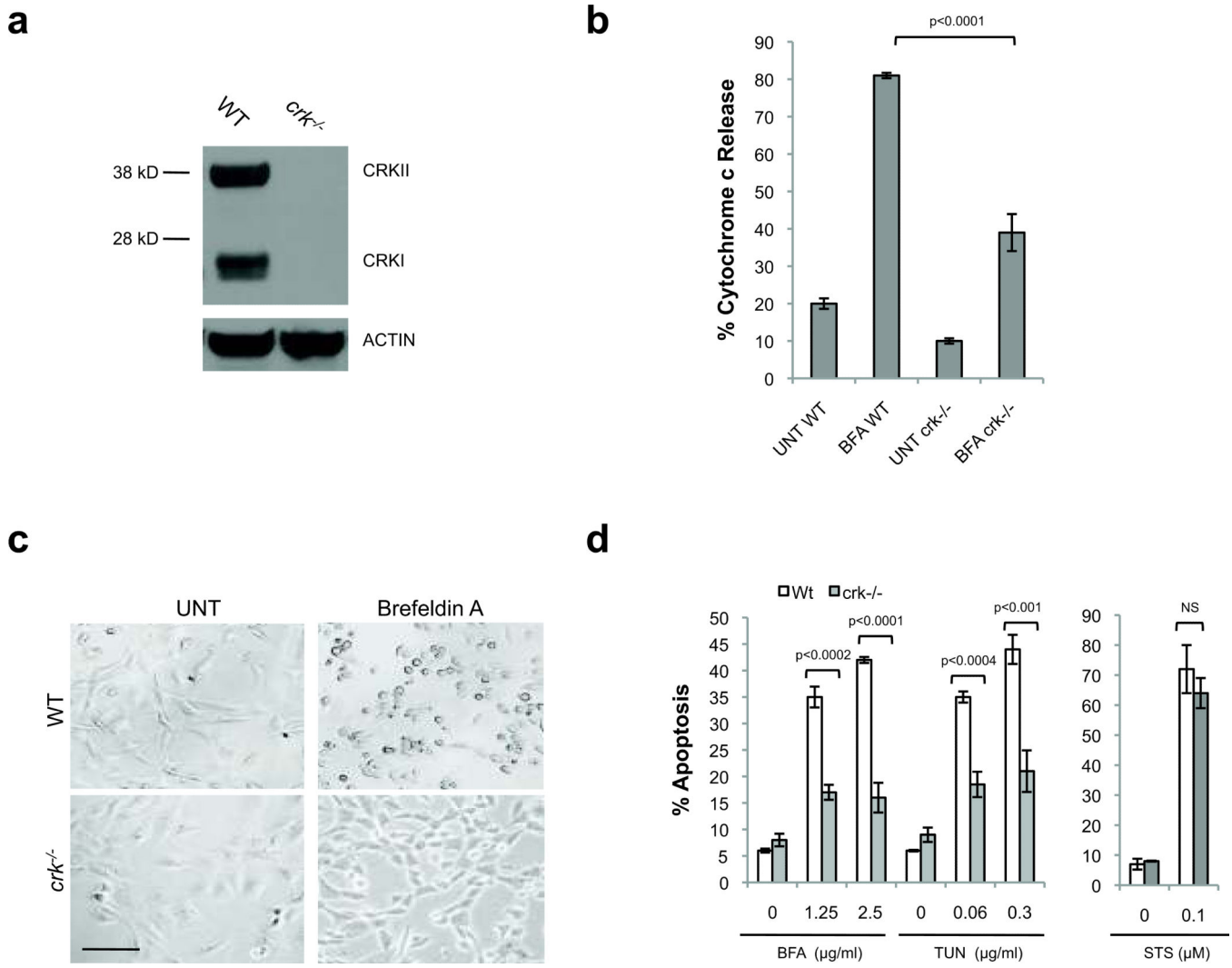

Figure 2. *Crk*$^{-/-}$ MEFs are significantly resistant to ER stress-induced apoptosis

(**a, b**) 18h BFA (2.5 µg/ml)-treated *crk*$^{-/-}$ MEF S100 contains significantly less C*c*RA in comparison to 18h BFA-treated (2.5 µg/ml) wild-type MEF S100. n=3, error bars = sd. (**c**) *crk*$^{-/-}$ MEFs are visually resistant (phase contrast) to ER stress-induced apoptosis (BFA 2.5µg/ml). Scale bar, 100µm. (**d**) *crk*$^{-/-}$ MEFs are strongly resistant to BFA and TUN-induced apoptosis, but equally sensitive to staurosporine (STS), in comparison to wild-type MEFs. n=3, error bars = sd.

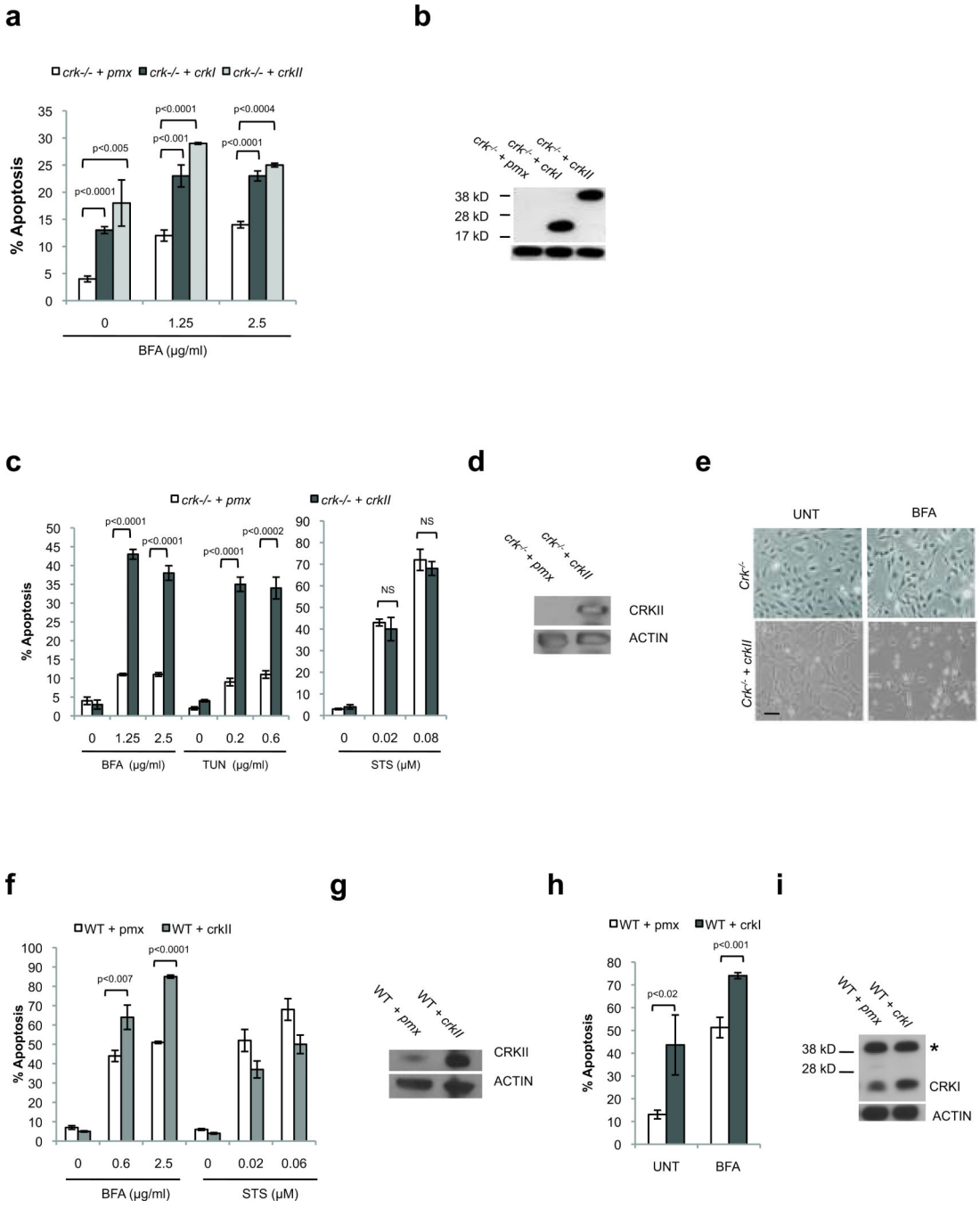

Figure 3. CRKI or CRKII restores sensitivity of *crk*$^{-/-}$ MEFs to ER stress-induced apoptosis

(**a, b**) Transient expression of CRKI or CRKII sensitizes *crk*$^{-/-}$ MEFs to BFA-induced apoptosis. n=3, error bars = sd. (**c-e**) Stable CRKII expression in *crk*$^{-/-}$ MEFs rescues sensitivity to 24h BFA- and 18h TUN-induced apoptosis, but does not change sensitivity to STS-induced apoptosis. n=3, error bars = sd. Scale bar, 100µm. (**f, g**) Stable overexpression of CRKII in wild-type MEFs further increases sensitivity to 18h BFA-induced apoptosis. n=3, error bars = sd. (**h, i**) Transient overexpression of CRKI sensitizes WT MEFs to 18h BFA (1.25 µg/ml)-induced apoptosis. n=3, error bars = sd.

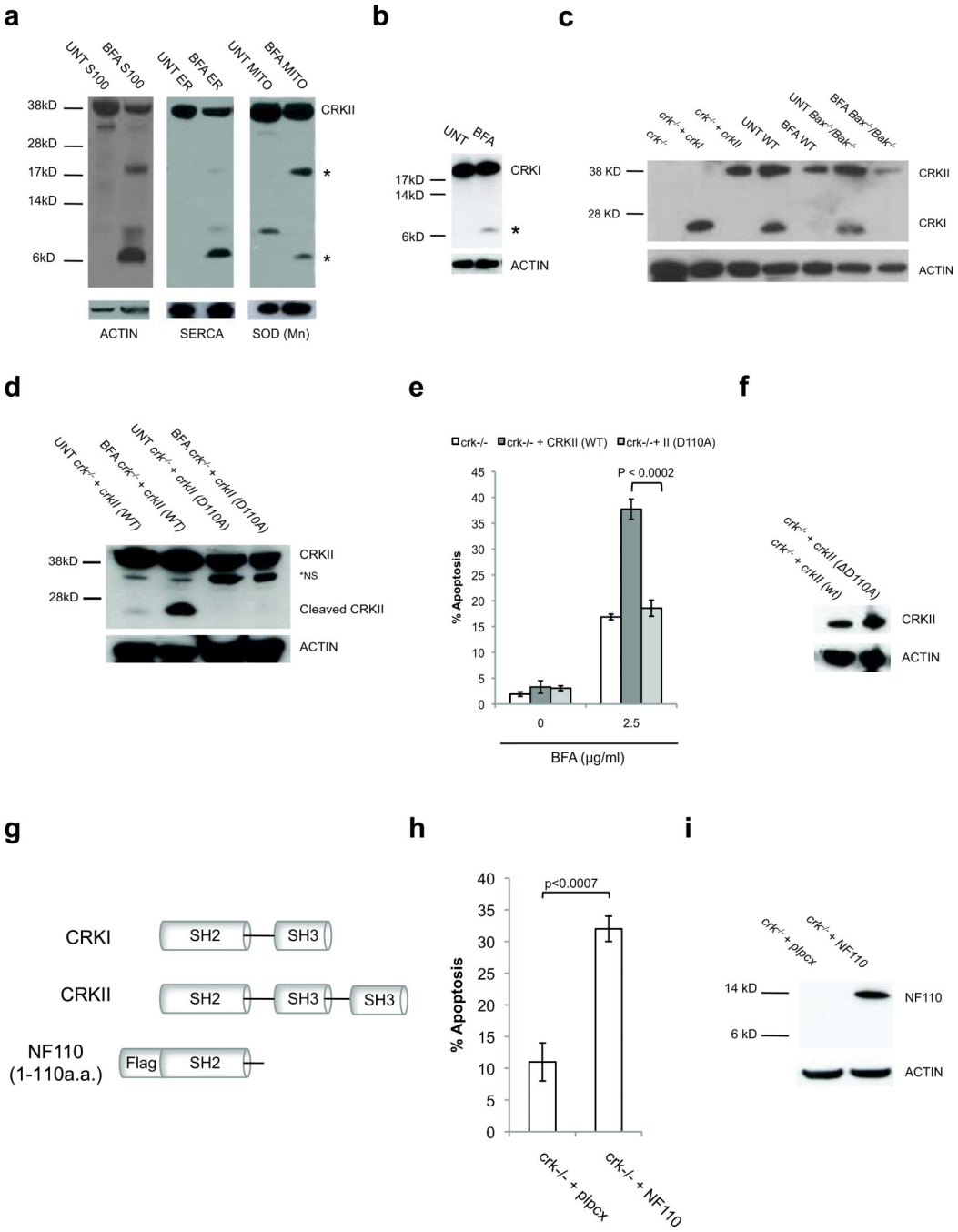

Figure 4. CRK is proteolytically cleaved into an apoptotic signal upon irremediable ER stress

(**a**) Upon 24h BFA (2.5 μg/ml) treatment of *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs, full-length CRKII is depleted in the cytosol and at the ER. CRKII-specific fragments (*) appear in the cytosol, ER, and mitochondria. (**b**) Transiently expressed CRKI is also cleaved upon 24h BFA (2.5 μg/ml) treatment in *crk*$^{-/-}$ MEFs. * = CRKI-specific fragment. (**c**) Loss of full-length, endogenous CRKI and CRKII observed upon 18h BFA treatment of WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. (**d**) Upon ER stress, CRK is cleaved at D110. Mutation of this site (D110A) in CRKII prevents cleavage following 24h 2.5 μg/ml BFA treatment in stably reconstituted in

crk$^{-/-}$ MEFs. (**e, f**) CrkII (D110A) is not able to rescue *crk*$^{-/-}$ MEF sensitivity to ER stress-induced apoptosis induced by 24h 2.5 μg/ml BFA, in contrast to *crk*$^{-/-}$ MEFs stably expressing wild-type (WT) CRKII. n=3, error bars = sd. (**g**) Diagram of CRK (1-110a.a.) cleavage fragment (NF110) produced upon ER stress. (**h, i**) Transient expression of NF110 induces apoptosis independent of ER stress. n=3, error bars = sd.

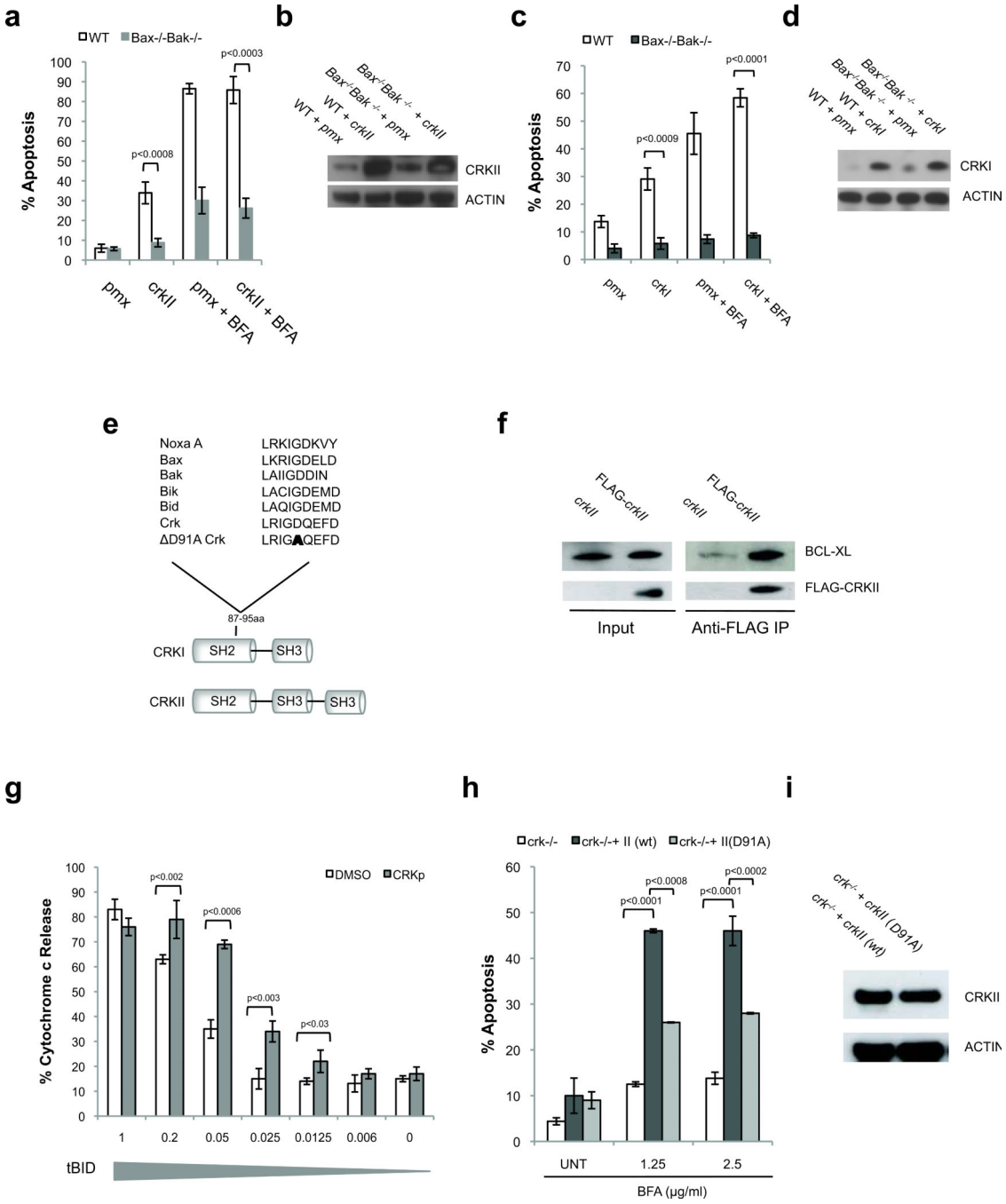

Figure 5. CRKII contains a putative BH3 domain and triggers BAX/BAK-dependent apoptosis

(a, b) CRKII and empty vector (pmx) were transiently overexpressed in WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs using retroviral infection. 24h post retroviral infection cells were treated with BFA (2.5 μg/ml) for an additional 24h and analyzed for Annexin-V expression by flow cytometry. n=3, error bars = sd. (**c, d**) CRKI and empty vector (pmx) were transiently overexpressed in WT and *Bax*$^{-/-}$*Bak*$^{-/-}$ MEFs. 24h post transfection cells were treated an additional 18h with BFA (2.5 μg/ml) and analyzed for Annexin-V expression by flow cytometry. n=3, error bars = sd. (**e**) The sequences of the putative BH3-only domain of CRK and the "BH3 domain"

point mutation D91A are aligned against BH3 domains of several known BH3-only proteins. (**f**) 293 cells were transiently transfected 24h with *Flag-crkII* or untagged *crkII*, then treated 14h BFA (1.25μg/ml). Lysates were incubated with FLAG-specific agarose beads. Beads were immunoblotted for endogenous BCL-XL. (**g**) Cytochrome *c* release from isolated Jurkat mitochondria incubated with decreasing doses of tBID and CRK BH3 domain peptide. n=3, error bars = sd. (**h, i**) Stable reconstitution of D91A crkII into $crk^{-/-}$ MEFs is significantly less effective at restoring ER stress-induced apoptosis (24h BFA treatment) in comparison to expression of wild-type *crkII*. n=3, error bars = sd.