## Title
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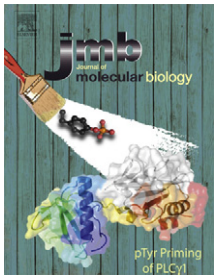


# A Regulated, Ubiquitin-Independent Degron in IκBα

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## Abstract

Whereas ubiquitin-dependent degrons have been characterized in some detail, how proteins may be targeted to ubiquitin-independent proteasomal degradation remains unclear. Here we show that IκBα contains an ubiquitin-independent degron whose activity is portable to heterologous proteins such as the globular protein GFP (*g*reen *f*luorescent *p*rotein) via a proteasome-dependent, ubiquitin-independent, non-lysosomal pathway. The ubiquitin-independent degradation signal resides in an 11-amino-acid sequence, which is not only sufficient but also required for IκBα's short half-life. Finally, we show that this degron's activity is regulated by the interaction with NFκB, which controls its solvent exposure, and we demonstrate that this regulation of the degron's activity is critical for IκBα's signaling functions.



Protein degradation may be mediated by the lysosome, an organelle packed with enzymes for the breakdown of diverse biomolecules, or the proteasome, a 20S oligomeric molecular machine [1]. Proteasomal protein degradation affords a high level of control via a 19S regulatory cap that ensures that only proteins tagged with K48-linked ubiquitin chains are degraded [1]. The sequences within proteins that contain ubiquitin acceptors (lysine, threonine, serine, and cysteine) and/or potentially recruit ubiquitin ligases and that therefore confer half-life control have been termed degrons [2].

A number of proteins have been identified to undergo degradation in the absence of ubiquitin, and unstructured sequences have been identified to mediate recognition with the proteasome [3–7]. In the case of uncomplexed IκBα, the C-terminal region is poorly structured and required for high turnover [8–13], which in turn was shown to be important for its signaling functions in controlling NFκB homeostasis [14] and signal responsiveness [8,15,16]. However, it has not been established whether IκBα contains an actual degron; that is, a sequence that confers short half-life to heterologous proteins has not been established.

### IκBα contains an ubiquitin-independent degron

To test whether IκBα high turnover could be transferred to a heterologous protein, we fused it to GFP (*g*reen *f*luorescent *p*rotein). We chose GFP because it is a long-lived, globular protein that is known to fold independently regardless of whether it is fused to other protein sequences [17]. Following transduction of the NFκB-deficient *crel*$^{-/-}$*nfkb1*$^{-/-}$*rela*$^{-/-}$ 3T3 cell line (referred to as *nfkb*$^{-/-}$ hereafter) with the recombinant retrovirus encoding the fusion protein, we observed GFP fluorescence in the cytoplasm of a fraction of cells (Fig. 1a). Following 30 min of treatment with the translation inhibitor cycloheximide (CHX), this fluorescence disappeared. However, co-treatment with the proteasome inhibitor MG132 recovered the cytoplasmic fluorescence. Appearance of nuclear fluorescence was deemed to be background, as even untransduced cells treated with MG132 acquired strong nuclear

 

fluorescence. These studies demonstrated that the short half-life control of IκBα could be transferred to the globular protein GFP and that proteolysis was sensitive to proteasome inhibitor.

There are two well-known intracellular protein degradation pathways in mammalian cells: the lysosomal pathway, which is generally non-selective, and the ubiquitin-proteasome system, which is selective via

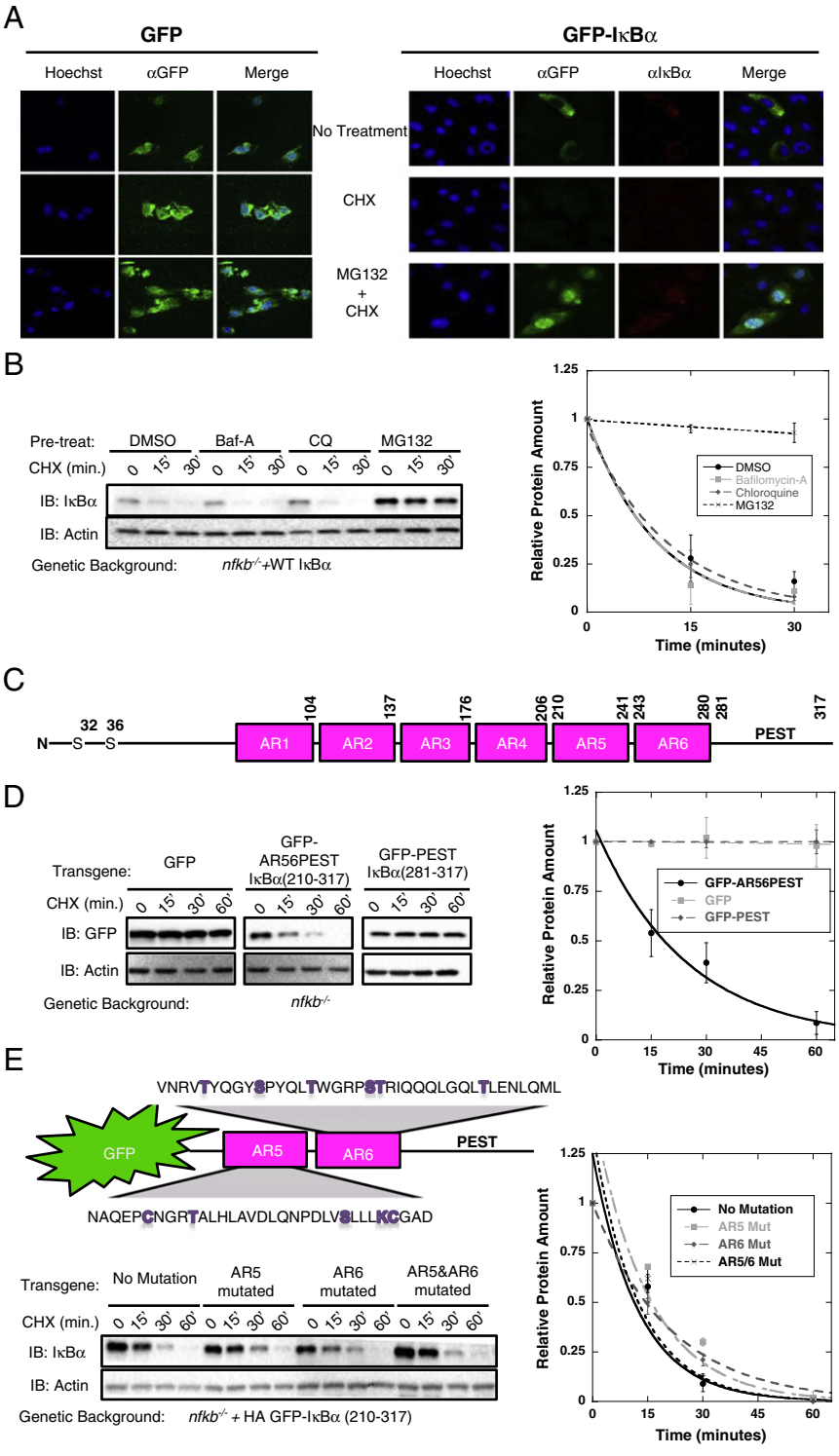


**Fig. 1** (*legend on next page*)

ubiquitin E3 ligases [1]. However, both may be inhibited by MG132 [18]. Pretreating IκBα-expressing *nfkb*$^{-/-}$ cells with the lysosomal inhibitors Bafilomycin-A and Chloroquine before administering cycloheximide (CHX) in a time course, we did not observe any decrease in IκBα turnover (Fig. 1b). Only with the MG132 control did we see a rapid increase in protein levels, confirming *in vitro* studies that free IκBα degradation is mediated by the proteasome [8,10].

To identify the molecular determinants of IκBα's short half-life, we engineered a number of recombinant forms respecting IκBα's key structural elements (Fig. 1c). We sought to determine if the C-terminal portion of IκBα shown to be required for short IκBα half-life [8] was a sufficient determinant for degradation and thus fused it to GFP, expressing the resulting GFP-IκBα (210-317) in *nfkb*$^{-/-}$ cells. To measure the approximate half-life, we again used cycloheximide time courses and immunoblotted for IκBα. Whereas the GFP control construct was stable during the 60-min time course, the fusion protein showed a half-life of less than 15 min (Fig. 1d). The C-terminal PEST sequence had been suspected to play a role in IκBα turnover. However, we found that the PEST domain of IκBα was not sufficient to grant degradation and in fact was stable during a 60-min time course (Fig. 1d).

It was previously shown that the lysines of free IκBα are not required for free IκBα degradation [8]. However, ubiquitin conjugations on other amino acids such as cysteine, serine, and threonine via thiodiester and hydroxyester linkages have been reported to mediate proteasomal degradation [19]. To examine whether these atypical ubiquitin acceptors might play a role in the degradation of IκBα, we first assayed for ubiquitin modification using a sensitive assay of overexpressed HA-ubiquitin in HEK293T cells; we found that the GFP-AR56PEST IκBα construct showed no more than GFP background signals unlike the full-length IκBα construct (Fig. S1). We then mutated all lysines, cysteines, serines, and threonines in ankyrin repeats 5 and/or 6 within this construct to alanine (lysine was mutated to arginine) and found that, following retroviral transduction into *nfkb*$^{-/-}$ cells, cycloheximide time courses revealed no change in the half-life of the mutants (Fig. 1e).

In sum, our results confirm and extend previous studies [8,10] indicating that the IκBα C-terminus contains a degron, a peptide sequence (other than PEST) that confers ubiquitin-independent degradation by the proteasome.

## An 11-amino-acid degron within ankyrin repeat 6

To identify the peptide sequence conferring degradation of IκBα, we generated mutated variants of the fifth and sixth ankyrin repeats (AR5 and AR6) using the GFP fusion expressing *nfkb*$^{-/-}$ cell system. In cycloheximide time courses (Fig. 2a), GFP-IκBα (AR5: 210-241) showed very similar degradation kinetics to the stable GFP control and PEST domain of IκBα (Fig. 1c). However, the GFP-IκBα (AR6: 243-280) construct showed rapid degradation analogous to the degradation of the complete C-terminus of IκBα (281-317). The quantitated data showed that the half-life of GFP-IκBα (281-317) and that of GFP-AR5 IκBα (210-241) had half-lives greater than 2 h while the GFP-IκBα (AR6: 243-280) had a

**Fig. 1** IκBα contains an ubiquitin-independent degron.(a) Immunofluorescence for GFP and IκBα of *nfkb*$^{-/-}$ 3T3 cells previously described [14] expressing GFP or GFP-IκBα following retroviral transduction with pBabe-puro and puromycin selection. Cells were left untreated, treated with 10 μg/mL cycloheximide (CHX; Calbiochem) for 30 min, or pretreated with 10 μM MG132 (Sigma) for 60 min then treated with 10 μg/mL CHX for 30 min. Cells were fixed in 4% PFA (Electron Microscopy Sciences) on glass coverslips (Fisher) for 10 min at room temperature. Slides were blocked with 5% Normal Goat Serum and 0.2% Triton-X100 in PBS and stained with primary antibodies sc-9996 mouse-anti-GFP at 1:200 dilution and sc-371 rabbit-anti-IκBα at 1:200 dilution overnight at 4 °C. Secondary antibodies goat-anti-mouse Alexafluor-488 and goat-anti-rabbit Alexafluor-568 (Life Technologies) were used at 1:1000 at room temperature for 1 h. Images were acquired on an Axio Observer Z1 inverted microscope (Carl Zeiss Microscopy GmbH, Germany) with a 40×, 1.3 NA oil-immersion objective to an Orca Flash sCMOS camera (Hamamatsu, Japan) using ZEN imaging software.(b) Lysosomal inhibitors do not extend free IκBα half-life. Immunoblot for IκBα (C-terminal antibody) of whole cell extracts prepared from *nfkb*$^{-/-}$ cells stably expressing WT IκBα, pretreated for an hour with DMSO, Bafilomycin-A, Chloroquine, or MG132 and then treated with CHX for indicated periods of time. Cell extracts made with RIPA buffer were separated on 4–15% gradient SDS-PAGE (Bio-Rad) and transferred to PVDF membrane. IκBα was probed with sc-371 or sc-203 then followed by HRP conjugate. Quantification of immunoblots was performed with ImageJ. The right panel shows quantification of the data.(c) Schematic of the IκBα protein. The signal response domain (SRD), the ankyrin repeat domains (ARD), and the PEST domain are indicated. IKK phosphorylation sites are indicated, as well as the amino acid residues of the six ankyrin repeats (AR).(d) Immunoblot for GFP (sc-9996; Santa Cruz Biotechnology) of whole cell extracts prepared from CHX-treated *nfkb*$^{-/-}$ cells expressing GFP (GFP), GFP-IκBα (210-317) or GFP- IκBα (281-317). Right panel shows quantification of three experiments, and error bars indicate standard deviation.(e) Upper panel: schematic of IκBα indicating the amino acids in the fifth and sixth repeats, and atypical ubiquitin acceptors are highlighted. Lower left panel shows *nfkb*$^{-/-}$ cells expressing either non-mutated IκBα (210-317) or IκBα (210-317) where all ubiquitin acceptors are mutated within AR5, within AR6, or within both AR5 and AR6 that were treated with CHX and protein levels were detected by Western blot using an antibody directed against the C-terminus of IκBα. Lower right panel shows quantification of three experiments, and error bars indicate standard deviation.

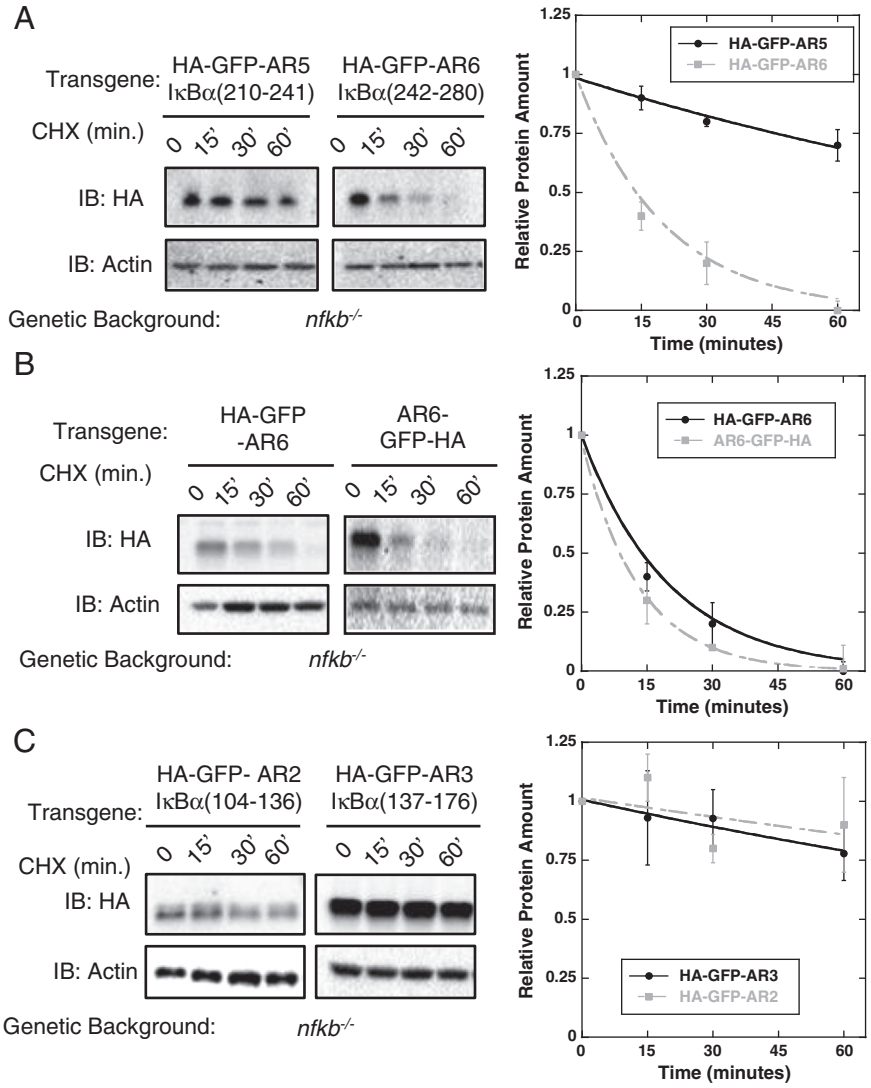


**Fig. 2.** The degron resides in ankyrin repeat 6. (a) Immunoblot for HA with antibody 16b12 (Covance), followed by HRP conjugate, of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing AR5 [IκBα (210-241)] or AR6 [IκBα (242-280)] treated with CHX, as previously described. Right panel shows quantification of experiments, and error bars indicate standard deviation representative of three experiments. (b) Immunoblot for HA of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing AR6 [IκBα (242-280)] with the HA-GFP tag at either the N-terminus or the C-terminus of the protein, treated with CHX. Right panel shows quantification of experiments, and error bars indicate standard deviation and are representative of at least three experiments. (c) Immunoblot for HA of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing AR3 [IκBα (137-176)] or the AR2 [IκBα (104-136)] treated with CHX. Right panel shows quantification of three experiments, and error bars indicate standard deviation.

half-life of around 15 min. These findings indicate that the degron of IκBα is in its sixth ankyrin repeat.

Previous work established that the short half-life of thymidine synthase depends on an ubiquitin-independent degron, which must be located at its very N-terminus, as blocking the N-terminus with a His tag caused protein stabilization [20]. To examine whether the IκBα degron function depended on its C-terminal location, we engineered a construct in which the HA-GFP tag was fused to the C-terminus rather than to the N-terminus of the 243-280 region (AR6) (Fig. 2b). Cycloheximide time course data show that the degron's location does not affect its activity.

The C-terminal region of IκBα is known to be incompletely folded when not bound to NFκB [9,21]. We asked whether the internal ankyrin repeats AR2 and AR3 may be harboring latent degrons also, hidden within the folded ARD of AR1–AR4. As three ARs are required for stable folding of an ARD [22], we examined AR2 and AR3 in isolation but neither

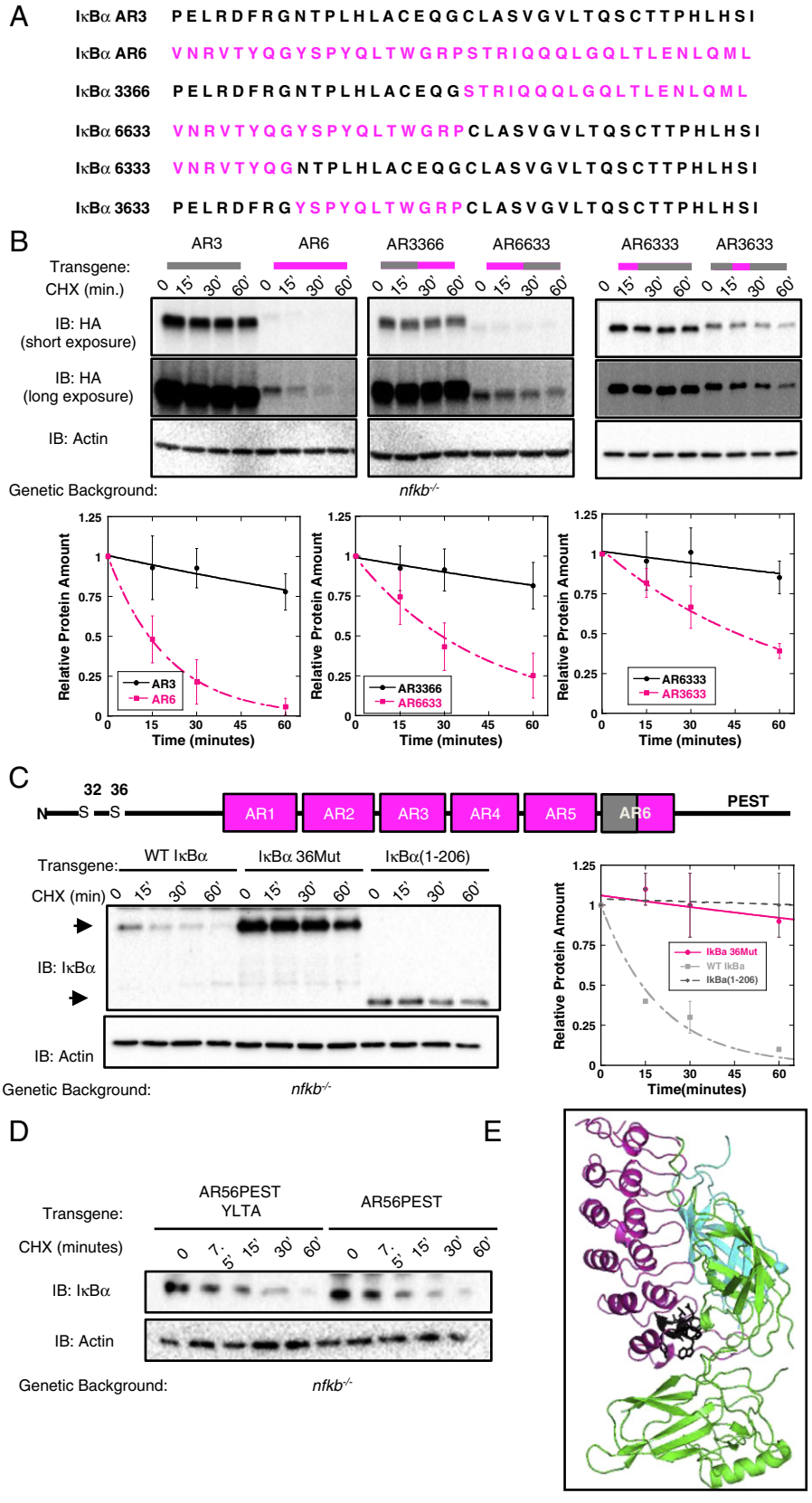


**Fig. 3** (*legend on next page*)

caused similar degradation of GFP after a 60-min exposure to CHX (Fig. 2c). These results indicate that there are specific degron sequences within AR6 that are not present in other ARs of IκBα.

To determine in more detail which segment of AR6 is responsible for free IκBα degradation, we exploited the functional differences between the structurally homologous AR3 and AR6 and generated chimeric constructs (Fig. 3a). First, we fused the first half of AR6 to the second half of AR3 (mutant 6633) and vice versa (mutant 3366). Within the *nfkb*$^{-/-}$ cell system, the 3366 mutant showed a half-life greater than 60 min while the 6633 mutant had a half-life of 28 min similar to the full ankyrin repeat 6 (Fig. 3b). These results suggested that the first half of AR6 (IκBα 243-262) contains the IκBα degron activity.

In order to further identify the amino acid sequence responsible for IκBα degradation, we integrated either the first or the second quarter of AR6 into AR3 (mutant 6333 or 3633; Fig. 3a). Within the *nfkb*$^{-/-}$ cell system, we observed a relatively short half-life for the 3633 mutant (Fig. 3b) while the 6333 mutant was more stable. These results show that primary IκBα degron activity is mediated by residues 251–262. We made further attempts to locate specific amino acids critical for its activity. Given that 3 of the 11 residues are aromatics, we wondered whether they may be involved in ubiquitin-independent proteasomal targeting. However, mutating Y251A, Y254A, and W258A, singly or in combination, did not result in a longer half-life (Fig. S2). As the 3633 mutant did not show a half-life as short as the full-length AR6 or the 6633 mutant, we conclude that additional residues within the first half of AR6 may also contribute to the degron's activity.

Our studies thus far identified the first half of ankyrin repeat 6 of IκBα as sufficient for triggering degradation of the heterologous reporter protein GFP. Now we asked whether this sequence might also be required for IκBα degradation in the context of the full-length protein. We engineered a mutant form of IκBα that had its sixth ankyrin repeat replaced with the 3366 chimeric repeat (Fig. 3c), stably transduced this construct, as well as wild-type IκBα and the long-lived IκBα (1-206) controls into *nfkb*$^{-/-}$ cells, and treated these cell lines with cycloheximide. Immunoblotting for IκBα revealed a stabilization of IκBα in the 36Mut that was similar to the IκBα (1-206) control (Fig. 3c). These results demonstrate that the first half of AR6 contains a degron sequence that is both sufficient for degrading heterologous proteins and required for the degradation of free IκBα. We conclude that the degron of IκBα has a non-redundant function in IκBα degradation.

## The degron must be regulated to provide for proper control of NFκB signaling

The degron peptide sequence is located within the previously characterized incompletely folded C-terminus of IκBα [21], and deviations from the AR consensus were noted. Indeed, mutations Y254L and T257A toward the AR consensus increased IκBα foldedness and *in vivo* half-life [9]. Interestingly, testing these mutations within the isolated AR6 in our system did not prolong the protein half-life (Fig. 3d), supporting the notion that these mutations did not delete the degron itself but decreased its activity by increasing the region's foldedness. Similarly, the degron's solvent exposure is obscured when IκBα is bound to NFκB [21,23] (Fig. 3e), correlating with a long half-life [11,14,24].

We asked whether regulating the degron's activity is in fact important for NFκB signaling. To this end, we pursued a synthetic biology approach based on the IκB family member, IκBδ (Fig. 4a). This IκB is fully folded in solution, has a long half-life, and binds RelA:p50 with comparable affinity [25,26]. However, after attaching the IκBα signal-responsive domain SRD to the N-terminus and the IκBα AR6 degron to the C-terminus of IκBδ, the resulting chimera was destabilized in cells not only deficient in NFκB but also—unlike IκBα—containing NFκB (Fig. 4b).

Using an established kinetic model of the IκB-NFκB signaling module [14,27], we simulated TNF-induced NFκB activity mediated by wild-type IκBα or this mutant whose short half-life was identical whether free or bound with NFκB (Fig. 4c). When the

**Fig. 3** An 11-amino-acid sequence is sufficient and required.(a) Schematic detailing the amino acids in AR3 (in gray) and AR6 (in pink). The four chimeras (3366, 6633, 3633, and 6333) contain the indicated amino acids derived from the color-coded AR.(b) Immunoblot for HA of whole cell extracts prepared from *nfkb*$^{-/-}$ cells expressing the four chimeras treated with CHX. Bottom panel shows quantification of experiments, and error bars indicate standard deviation and are representative of at least three experiments.(c) The degron has a non-redundant role in IκBα degradation. Top, schematic of the 36Mut IκBα. Bottom, immunoblot for the N-terminus of IκBα of *nfkb*$^{-/-}$ cells expressing WT IκBα, IκBα 36Mut, or IκBα (1-206) treated with CHX. Right panel shows quantifications of three experiments, and error bars indicate standard deviation.(d) The degron does not rely on Y254 or T257, previously identified as maintaining the unfolded state (REF). Immunoblot for IκBα of whole cell extracts from *nfkb*$^{-/-}$ cells transduced with either GFP-IκBα (210-317) YLTA (whereby Y254 was mutated to leucine and T257 was mutated to alanine) or GFP-IκBα (210-317) treated with CHX for indicated times.(e) Location of the degron in the IκBα-NFκB complex. A ribbon diagram representation of the crystal structure of IκBα (pink) bound to NFκB (p50, cyan; p65, green). The location of the 11-amino-acid degron in black, depicted in a ball-and-stick representation. The figure was prepared using the PyMOL Molecular Graphics System, Version 1.7 Schrödinger, LLC.

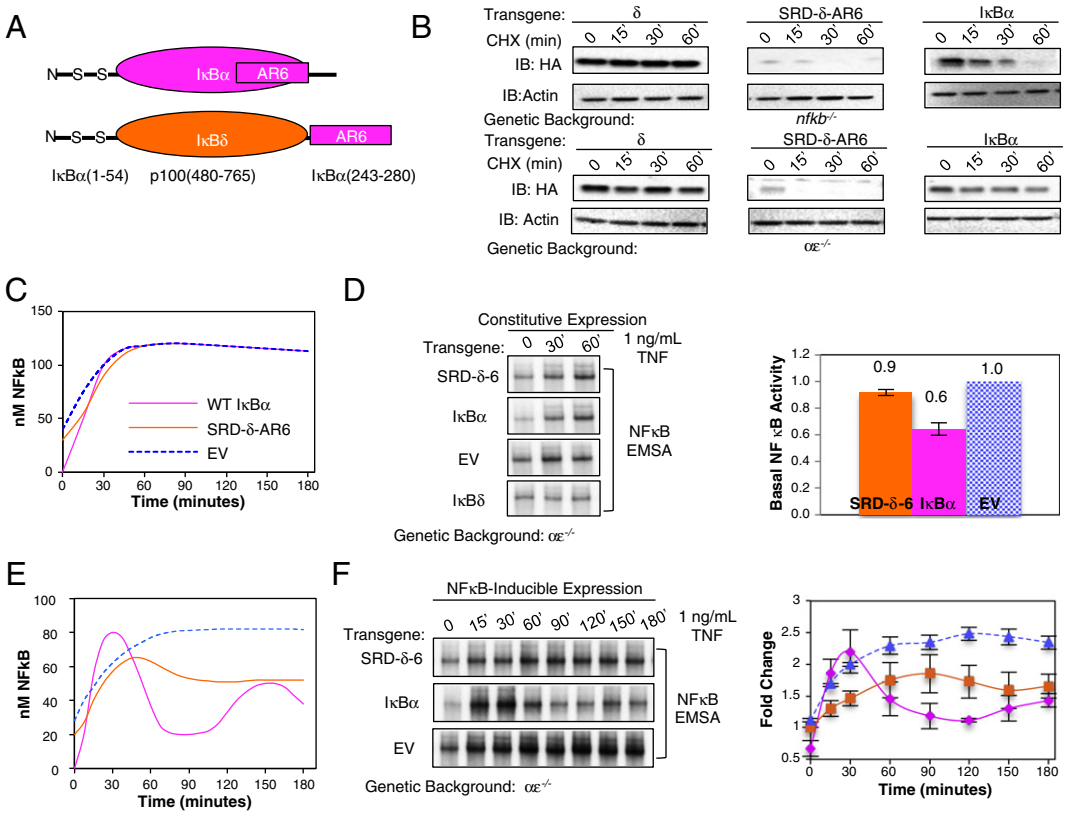


**Fig. 4.** Functional consequences of deregulating the degron's activity. (a) Schematic of mutants used. Highlighted within WT IκBα is the AR6 degron. SRD-δ-AR6 contains the signal response domain of IκBα (1-54), the ARD domain of p100 (480-765), and AR6 of IκBα (243-280). (b) Immunoblots for HA of whole cell extracts treated with CHX; top panel: *nfkb*$^{-/-}$ cells stably expressing IκBδ, SRD-δ-AR6, or WT IκBα. Bottom panel: top panel: $\alpha\varepsilon^{-/-}$ cells stably expressing WT IκBα ARD of p100, SRD-δ-AR6, or WT IκBα. (c) Computational simulations of NFκB activity in $\alpha\varepsilon^{-/-}$ cells expressing WT IκBα (pink), SRD-δ-AR6 (orange), and empty vector (EV) (dotted blue), treated with TNF. The Web model of the IκB-NFκB signaling module (http://www.signalingsystems.ucla.edu/models-and-code/, version 1.2) was used. Parameters governing mRNA half-life and synthesis were halved to simulate the NFκB control by the retrovirally expressed IκBα, and IKK (stimulation phase) was set to 40 nM. To simulate SRD-δ-AR6, the NFκB-bound IκBα degradation rate equal to the unbound IκBα degradation rate, and to simulate EV, we set the IκBα translation rate to zero and the NFκB concentration to 85 nM. (d) EMSA of NFκB activity in $\alpha\varepsilon^{-/-}$ cells expressing WT IκBα, SRD-δ-AR6, empty vector (EV), and IκBδ following stimulation with 1 ng/ml TNF (Roche). EMSAs were performed as previously described (32) and quantitated using a phosphoimager (Molecular Dynamics) and the NFY EMSA as a normalization control. Right panel: quantification of basal levels of NFκB in $\alpha\varepsilon^{-/-}$ cells expressing WT IκBα, SRD-δ-AR6, and empty vector (EV), in three experiments, and error bars indicate standard deviation. (e) Computational simulations of NFκB activity in $\alpha\varepsilon^{-/-}$ cells with NFκB feedback dynamics expressing WT IκBα in pink, SRD-δ-AR6 in orange, and empty vector (EV) in dotted blue, treated with TNF. (f) EMSA of NFκB activity in $\alpha\varepsilon^{-/-}$ cells expressing WT IκBα, SRD-δ-AR6, empty vector (EV), and IκBδ all in a vector derived from murine Moloney virus under the control of an NFκB-inducible promoter. Right panel shows quantifications of three experiments, and error bars indicate standard deviation.

half-lives were long, little NFκB activation was seen as the resulting IκB overexpression functioned as a transdominant inhibitor; when the half-lives were short, signaling was recovered; however, the basal NFκB activity was predicted to be elevated.

Using these IκB variants, we examined their effects on TNF-inducible NFκB activity. As expected, the fully stable IκB functioned as a transdominant inhibitor to suppress NFκB activation, compared to the IκBα positive control. In contrast, the destabilized chimera did allow for NFκB signaling, but we noted an elevated basal level of NFκB activity (Fig. 4d), as predicted. These results support the notion that NFκB control of IκBα half-life is critical for proper regulation of NFκB activity under basal and stimulus-induced conditions.

We then explored the effect of an unregulated degron on NFκB signaling dynamics that are largely shaped by the NFκB-IκBα negative feedback loop. Kinetic model simulations predicted that, while the IκB harboring a deregulated degron may provide some degree of post-induction attenuation, the extent and timing is defective, such that oscillations normally seen with wild-type IκBα cannot be

sustained (Fig. 4e). We tested these predictions with a retroviral construct that expresses the IκB variants under the control of five κB sites. Indeed, even with NFκB feedback, the unregulated degron does not recapitulate IκB's function (Fig. 4f). These data suggest that NFκB control of the IκB degron is essential for achieving the characteristically oscillatory NFκB signaling dynamics.

In this study, we have reported the identification and characterization of a signal sequence that triggers the ubiquitin-independent, proteasome-dependent degradation of IκBα. Our work clarifies that the so-called PEST region of IκBα is not a degradation signal for IκBα (although PEST sequences are generally thought to be responsible for protein turnover [28]) and that the lack of foldedness and solvent exposure is required but not sufficient for ubiquitin-independent degradation. Indeed, the identification of a specific sequence suggests new avenues for studying the biophysical basis of ubiquitin-independent degradation by the proteasome.

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**Author Contributions**: A.H. and K.T.F. designed experiments. K.T.F. generated and analyzed the experimental data, assisted by R.L. and K.A.N. R.F. generated the inducible construct. A.H. and K.T.F. wrote the manuscript.

**Conflict of Interest**: The authors declare no conflict of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.07.008.

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