# Activation of Transcription Factor NF-κB Requires ELKS, an IκB Kinase Regulatory Subunit

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**The nuclear factor–kappa B (NF-κB) family of transcription factors plays a seminal role in inflammation, apoptosis, development, and cancer. Modulation of NF-κB–mediated gene expression in response to diverse signals is coordinated by the IκB kinase (IKK) complex. We identified ELKS, an essential regulatory subunit of the IKK complex. Silencing ELKS expression by RNA interference blocked induced expression of NF-κB target genes, including the NF-κB inhibitor *IκBα* and proinflammatory genes such as *cyclo-oxygenase 2* and *interleukin 8*. These cells were also not protected from apoptosis in response to cytokines. ELKS likely functions by recruiting IκBα to the IKK complex and thus serves a regulatory function for IKK activation.**

The transcription factor NF-κB coordinates inducible gene expression in many cell types during fundamental biological processes, including the immune response, apoptosis, oncogenesis, and development (*1*, *2*). Exposure of cells to a multitude of external stimuli, including the cytokines tumor necrosis factor–α (TNF-α) and interleukin 1 (IL-1), initiates numerous and diverse intracellular signaling cascades, most of which activate the IKK complex. The IKK complex contains a kinase that phosphorylates IκBα, an inhibitory protein of NF-κB that sequesters the transcription factor in the cytoplasm. Phosphorylation triggers ubiquitination and degradation of IκBα (*3*, *4*), which then allows the release, modification, and translocation of NF-κB into the nucleus to activate the transcription of target genes.

IKK activity is associated with a multiprotein complex that has a high molecular weight (700 to 900 kD) (*5–7*). The complex contains several subunits, including two highly homologous catalytic kinases, IKK1/α (IKK1) and IKK2/β (IKK2), and an essential regulatory subunit, NEMO [NF-κB Essential Modulator, also known as IKKγ, IKK Associated Protein 1 (IKKAP1), and FIP-3 (type 2 adenovirus E3–14.7-kD interacting protein)] (*8–13*). Each kinase contains a catalytic domain at its N terminus and predicted protein-protein interaction motifs, including helix-loop-helix and a leucine zipper, at its C terminus. NEMO, which maintains a stoichiometric functional IKK complex and is required by upstream signals to activate IKK activity, also contains coiled-coil and leucine zipper motifs. An additional heterocomplex, cdc37-hsp90, has also been implicated in chaperoning the IKK complex to the activated TNF receptor (*14*).

We identified by gel filtration analysis and tandem mass spectrometry a 105-kD protein, ELKS, that copurified with an IKK complex that was immunoprecipitated with IKK2 antibodies from a human cell line (HeLa) treated with TNF-α (*12*, *15*) [The name ELKS is derived from the relative abundance of its constitutive amino acids: glutamic acid (E), leucine (L), lysine (K), and serine (S) (*16*)]. ELKS has previously been identified as a ubiquitous protein of unknown function (*16*, *17*). Secondary structure prediction of the ELKS sequence indicates coiled-coil motifs throughout the protein and a leucine zipper at the C terminus (fig. S1A). The IKK complex with the high molecular weight of 700 to 900 kD was also present in anti-ELKS immunoprecipitates from HeLa cells treated with TNF-α, as determined by gel filtration and immunoblot analysis (Fig. 1A).

Immunoprecipitation of ELKS from lysates of HeLa cells either treated with TNF-α or untreated contained endogenous IKK1, IKK2, and NEMO (Fig. 1B). Additionally, antibodies to either IKK1, IKK2, or NEMO immunoprecipitated ELKS from lysates of cells that were treated with TNF-α or untreated (Fig. 1C). Association of ELKS with the IKK complex was also identified in multiple cell types, including human embryonic kidney cells (293T) and human T-lymphocyte cells (Jurkat) (fig. S1B). Inducible IKK activity was also detected in ELKS that was immunoprecipitated from lysates of HeLa cells treated with TNF-α (Fig. 1D). Similar results were obtained when epitope (Myc)–tagged ELKS (ELKS-Myc) was transiently expressed in transfected 293T cells (fig. S1C).

Increasing amounts of ELKS protein were immunoprecipitated when increasing amounts of ELKS antibody were used (Fig. 1E, top), indicating that ELKS was progressively depleted from lysates of HeLa cells that were untreated or treated with TNF-α (Fig. 1E, bottom). Endogenous IKK1, IKK2, and NEMO were also depleted concomitantly regardless of TNF-α treatment (Fig. 1E, bottom), suggesting that ELKS, IKK1, IKK2, and NEMO are stoichiometric integral components of the functional high–molecular weight IKK complex.

To determine whether ELKS specifically associates with each of the three well-characterized components of the IKK complex, ELKS-Myc was transiently expressed with either epitope-tagged IKK1 [hemagglutinin (HA)], IKK2 (Flag), or NEMO [Glu-Glu (EE)] in 293T cells. Each component immunoprecipitated with ELKS-Myc from cell lysates (Fig. 1, F to H). The multiple ELKS bands observed may be alternatively processed forms or degradation products of transiently expressed ELKS-Myc. ELKS and NEMO also coimmunoprecipitated from IKK1$^{-/-}$ IKK2$^{-/-}$ double knockout mouse embryonic fibroblast (MEF) cells, indicating that the association of ELKS and NEMO is independent of IKK1 and IKK2 (Fig. 1I). Both immunofluorescence and biochemical fractionation revealed that ELKS is localized in the cytoplasm where the IKK complex resides (fig. S1, D and E).

To address whether ELKS is essential for IKK activity and NF-κB activation, ELKS expression was reduced by RNA interference. A lentiviral vector was generated to synthesize ELKS small interfering RNA (LV-siELKS) by the human H1 polymerase III promoter (*18–20*) (Fig. 2A). Five days after infection, lysates from HeLa cells transduced with either LV-siELKS or a control lentiviral vector that synthesized siRNA against green fluorescent protein (LV-siGFP) were immunoprecipitated with the antibody to ELKS, followed by immunoblot analysis. Several spliced variant isoforms of ELKS were generated that encode proteins ranging from 948 to 1116 amino acids (*21*). The N-terminal ELKS antibody was generated against a peptide common to all isoforms. LV-siELKS reduced the expression of two major ELKS isoforms in HeLa cells as compared with expression in control cells. Expression of IKK1, IKK2, NEMO, and NF-κB (composed of p65 and p50) remained stable in the absence of ELKS (Fig. 2A). ELKS silencing caused a loss and delay of IκBα phosphorylation and degradation in response to TNF-α and IL-1, whereas IκBα from control cells displayed a normal profile of phosphorylation, degradation, and resynthesis in response to cytokines (Fig. 2B). Lysates from LV-siGFP or LV-siELKS transduced HeLa cells that were treated with TNF-α were immunoprecipitated with an antibody to NEMO and were assessed for IKK activity. In the absence of ELKS, IKK displayed reduced ability to phosphorylate an exogenous substrate glutathione *S*-transferase (GST)–IκBα, residues 1 to 54 [GST-IκBα(1–54)] (a fusion protein of GST and IκBα) (Fig. 2C).

Failure to activate IKK activity and degrade IκBα resulted in the loss of DNA binding ac-

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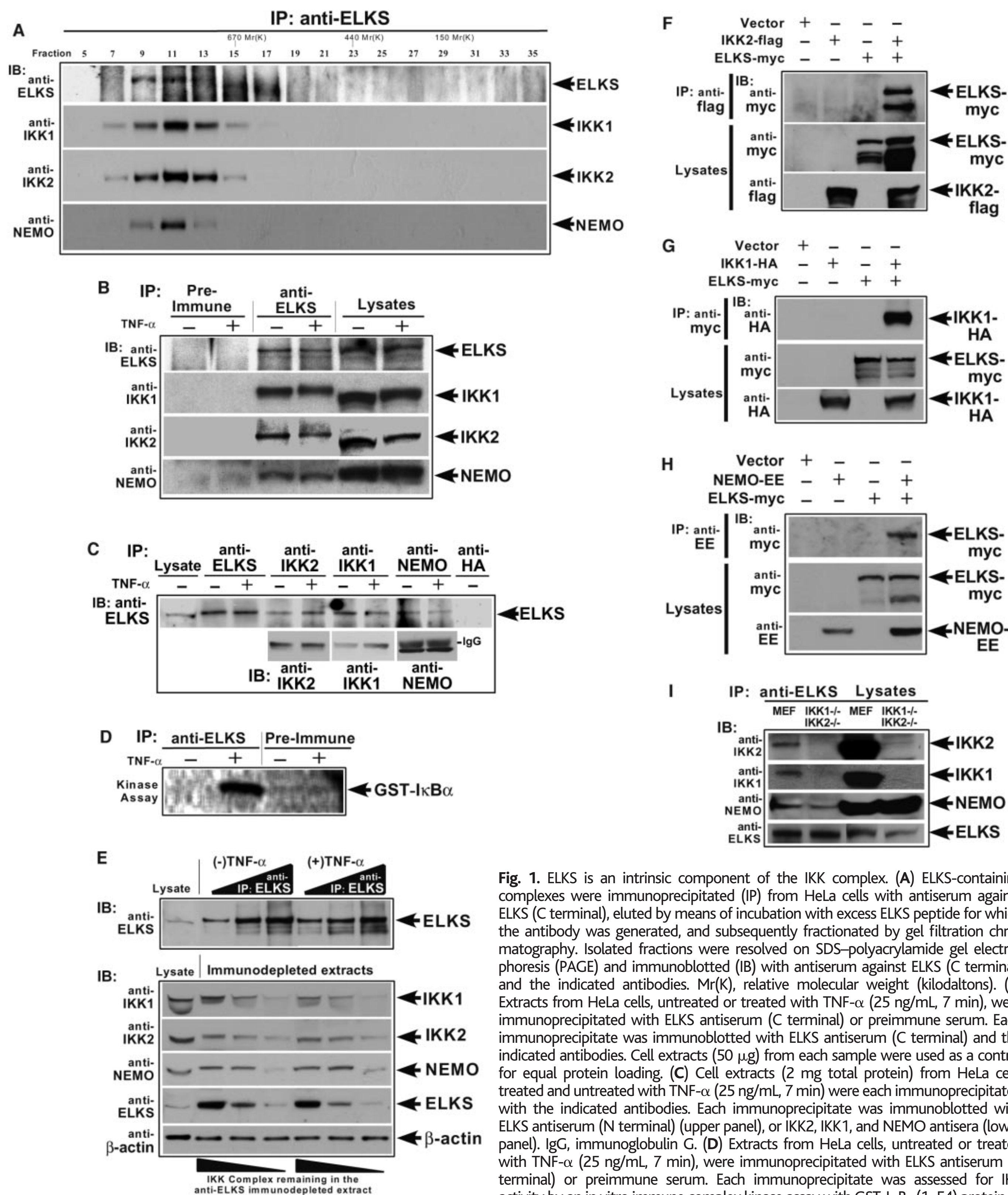


**Fig. 1.** ELKS is an intrinsic component of the IKK complex. (**A**) ELKS-containing complexes were immunoprecipitated (IP) from HeLa cells with antiserum against ELKS (C terminal), eluted by means of incubation with excess ELKS peptide for which the antibody was generated, and subsequently fractionated by gel filtration chromatography. Isolated fractions were resolved on SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotted (IB) with antiserum against ELKS (C terminal) and the indicated antibodies. Mr(K), relative molecular weight (kilodaltons). (**B**) Extracts from HeLa cells, untreated or treated with TNF-α (25 ng/mL, 7 min), were immunoprecipitated with ELKS antiserum (C terminal) or preimmune serum. Each immunoprecipitate was immunoblotted with ELKS antiserum (C terminal) and the indicated antibodies. Cell extracts (50 μg) from each sample were used as a control for equal protein loading. (**C**) Cell extracts (2 mg total protein) from HeLa cells treated and untreated with TNF-α (25 ng/mL, 7 min) were each immunoprecipitated with the indicated antibodies. Each immunoprecipitate was immunoblotted with ELKS antiserum (N terminal) (upper panel), or IKK2, IKK1, and NEMO antisera (lower panel). IgG, immunoglobulin G. (**D**) Extracts from HeLa cells, untreated or treated with TNF-α (25 ng/mL, 7 min), were immunoprecipitated with ELKS antiserum (C terminal) or preimmune serum. Each immunoprecipitate was assessed for IKK activity by an in vitro immune complex kinase assay with GST-IκBα(1–54) protein as substrate. (**E**) (top) Cell extracts (600 μg total protein) from HeLa cells, treated and untreated with TNF-α (25 ng/mL, 7 min), were each immunoprecipitated (IP) with increasing amounts of antibodies to ELKS (N terminal) (0.3 μl, 1 μl, or 3 μl). Each immunoprecipitate was immunoblotted with ELKS antiserum (N terminal). (bottom) After immunoprecipitation, 60 μg of the remaining IP lysate was immunoblotted with ELKS antiserum (N terminal) and the indicated antibodies to assess the residual amount of IKK complex still remaining in the lysate after immunoprecipitation with antibodies to ELKS. (**F** to **H**) Extracts from 293T cells that were transfected with (F) ELKS-Myc, IKK2-Flag, or vector alone; (G) ELKS-Myc, IKK1-HA, or vector alone; and (H) ELKS-Myc, NEMO-EE, or vector alone, as indicated, were immunoprecipitated with (F) antibodies to Flag, (G) antibodies to Myc, and (H) antibodies to EE. Immunoprecipitates and cell extracts (50 μg) from each treated sample were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. (**I**) Extracts from wild-type MEF or $IKK1^{-/-}$ $IKK2^{-/-}$ double knockout MEF cells were immunoprecipitated with ELKS (N-terminal) antiserum. Immunoprecipitates and cell lysates (50 μg) were immunoblotted with the indicated antibodies.

tivity of NF-κB to a radiolabeled consensus κB-oligonucleotide probe in response to TNF-α or IL-1 (Fig. 3A). In contrast, binding of an unrelated transcription factor, Oct-1, to its cognate DNA binding site was not impaired in these same extracts (Fig. 3A).

We also measured the effect of ELKS on the induction of the NF-κB targets *IκBα*, *cyclooxygenase 2* (*COX2*), and *interleukin 8* (*IL-8*), genes important in facilitating an immune response and inflammation (*22*). Normal levels of induced gene expression were observed in LV-siGFP cells treated with TNF-α as determined by real-time polymerase chain reaction (PCR). In contrast, gene expression was diminished in cells transduced with LV-siELKS (Fig. 3B). Expression levels of NF-κB independent genes, IκBβ and BRCA1, were unaffected in the absence of ELKS regardless of TNF-α treatment, indicating that the loss of gene expression was specific to NF-κB. In addition, luciferase reporter activity under the control of a human immunodeficiency virus (HIV)–NF-κB promoter was repressed, 4 hours post–cytokine induction in LV-siELKS cells (Fig. 3C). Another siRNA specifically targeting ELKS also diminished luciferase reporter activity in HeLa cells (base pairs 102 to 105 5′AACCAACAGTACGGGAGGGAG3′), suggesting that siELKS effects are due to loss of target gene product and not due to indiscriminate nontarget gene effects.

The physiological role of NF-κB's ability to protect cells from apoptosis was also examined in cells lacking ELKS. HeLa cells are normally resistant to apoptosis induced by TNF-α; however, suppression of NF-κB activity can sensitize cells to apoptosis (*23*–*25*). HeLa cells transduced with LV-siELKS displayed an increased sensitivity to apoptosis induced by TNF-α. Blebbing nuclei (Fig. 3D), poly(adenosine 5′-diphosphate–ribose) polymerase (PARP) cleavage, and pro-Caspase3 cleavage (Fig. 3E), indicative of apoptosis, were increased in HeLa cells transduced with LV-siELKS, compared with cells transduced with LV-siGFP. Thus, in cells lacking ELKS, the failure to activate NF-κB results in a lack of the survival gene expression necessary to protect the cells from apoptosis induced by TNF-α.

Previous studies have shown that the IKK complex associates with a small amount of IκBα at basal levels, and upon cytokine induction, IκBα is actively recruited to the high–molecular weight IKK complex as a target for phosphorylation (*26*, *27*). Consistent with these studies, IκBα was found in association with the IKK complex in HeLa cells treated with TNF-α or untreated (Fig. 4A and fig. S2). NEMO has been implicated in facilitating IκBα recruitment to the IKK complex upon IKK activation, although NEMO and IκBα do not directly interact unless IKK2 is present (*26*). Coimmunoprecipitation of epitope-tagged IκBα (Myc) and ELKS (V5) indicated that a specific interaction between ELKS and IκBα exists (Fig. 4B). Additionally, increasing amounts of IκBα were associated with increasing amounts of ELKS, but there was no concomitant increase of IKK activity (Fig. 4C), suggesting that ELKS association with IκBα is independent of IKK activity. This suggests that ELKS may directly recruit IκBα to the IKK complex. Consistent with this model, a reduction of ELKS protein levels in the cell not only diminished IKK activity but also delayed phosphorylation of IκBα (Fig. 2B).

We further identified an N-terminal deletion mutant (amino acids 1 to 134 deleted) of ELKS [ELKS(ΔN)] that facilitated the recruitment of IκBα and the induction of IKK and NF-κB activity in the absence of cytokine stimulation. Increased expression levels of ELKS(ΔN) resulted in enhanced NF-κB–dependent luciferase reporter gene activity in the absence of inducing stimuli, as compared with wild-type ELKS (Fig. 4E). As a control, another luciferase reporter under the control of the thymidine kinase promoter was unaffected (*28*). In contrast to wild-type ELKS, increased ELKS(ΔN) expression was associated with a decrease in endogenous IκBα interaction (Fig. 4D). Immunoblot analysis of cell lysates also revealed decreased levels of IκBα in these cells. In the absence of cytokine stimulation, kinase assays of ELKS(ΔN) immunoprecipitates revealed increased IKK activity with increasing amounts of ELKS(ΔN) (Fig. 4D) leading to the degradation of IκBα. This would account for decreased IκBα protein levels and enhanced NF-κB activity.

We propose that ELKS mediates an essential step in IKK activation. ELKS(ΔN) may exist in a conformational state that enhances IKK activity and IκBα recruitment in the absence of upstream stimulation. ELKS was initially identified from a papillary thyroid carcinoma as part of a translocation fusion protein with the receptor tyrosine kinase (RET), also known as tyrosine receptor kinase A (TRKA), tyrosine kinase domain (ELKS-RET) (*16*, *17*). Oligomerization of ELKS-RET chimeric proteins through the coiled-coil domain of ELKS dimerized the RET kinase domains. This resulted in the constitutive autophosphorylation of the RET tyrosine kinase, leading to the transforming function of ELKS-RET oncoproteins (*16*, *21*). Coordinated cascades of oligomerization events have been proposed as a mechanism of IKK activation (*29*–*31*). Considering the context in which ELKS was initially identified, ELKS may contribute an important oligomerization interface to members of the IKK complex as well as to other upstream or downstream regulators of NF-κB transcriptional activity, such as IκBα.

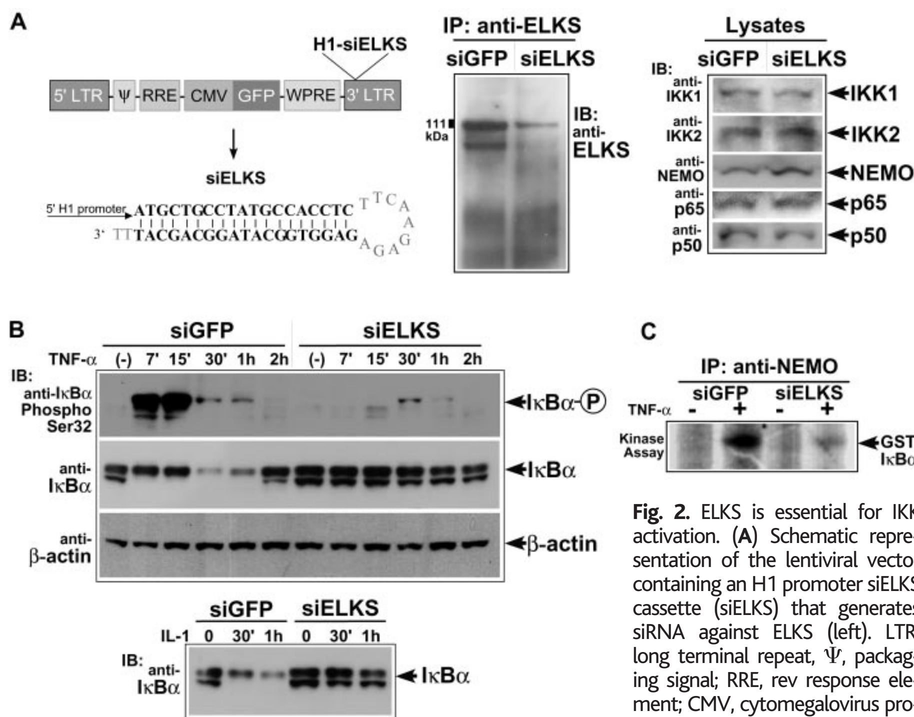


**Fig. 2.** ELKS is essential for IKK activation. (**A**) Schematic representation of the lentiviral vector containing an H1 promoter siELKS cassette (siELKS) that generates siRNA against ELKS (left). LTR, long terminal repeat, Ψ, packaging signal; RRE, rev response element; CMV, cytomegalovirus promoter; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. Extracts of HeLa cells transduced with LV-siGFP or LV-siELKS lentivirus were immunoprecipitated with antibodies to ELKS (against the N terminus), resolved on SDS-PAGE, and immunoblotted with antiserum against ELKS (N terminal) (middle). Cell lysates (50 μg) were immunoblotted with the indicated antibodies (right). (**B**) Extracts from LV-siGFP or LV-siELKS transduced HeLa cells, untreated or treated with TNF-α or IL-1 (25 ng/mL, for the indicated times), were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. (**C**) Extracts from HeLa cells (1 mg total protein) transduced with LV-siGFP or LV-siELKS, untreated or treated with TNF-α (25 ng/mL, 7 min), were immunoprecipitated with antibodies to NEMO. Each immunoprecipitate was assessed for IκB kinase activity by an in vitro immune complex kinase assay with GST-IκBα(1–54) protein as substrate.

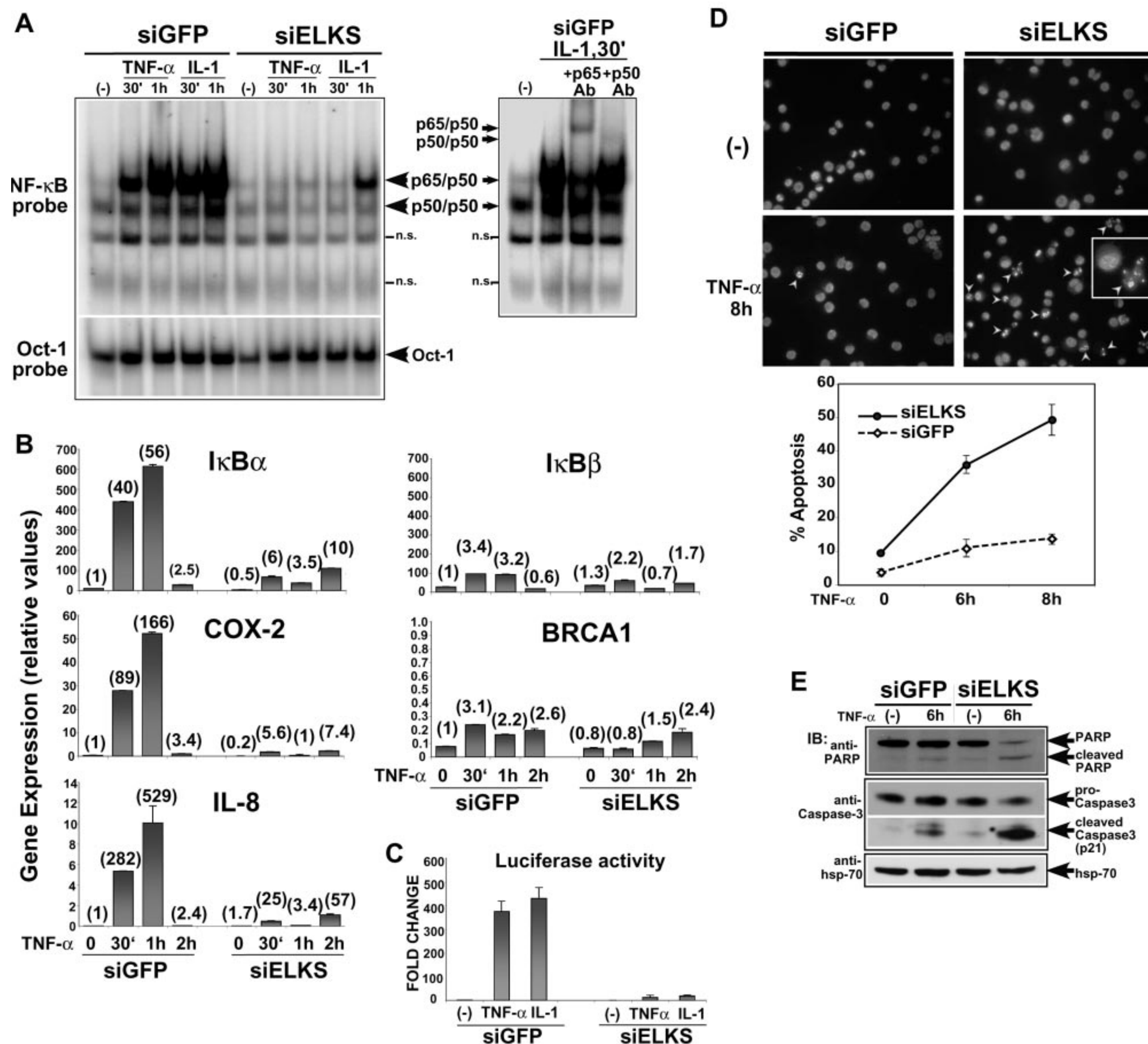


**Fig. 3.** ELKS is required for gene expression regulated by NF-κB and for protection from apoptosis induced by TNF-α. (**A**) (left, top) Cell extracts from HeLa cells transduced with LV-siGFP or LV-siELKS, untreated or treated with TNF-α or IL-1 (25 ng/mL), were assayed for the levels of NF-κB binding to an $^{32}$P end-labeled κB oligonucleotide probe by electrophoretic mobility shift assay (EMSA). (left, bottom) EMSA of the same cell extracts with an Oct-1 consensus probe for loading control. (right) Control showing NF-κB–specific binding with the same cell extracts incubated with antibodies (Ab) to p65 or p50. n.s., nonspecific. (**B**) RNA was extracted from HeLa cells transduced with LV-siGFP or LV-siELKS, which were untreated or treated with TNF-α (25 ng/mL) for the indicated time points. This RNA was used to prepare cDNA that was substrate for real-time PCR to quantify the mRNA levels of each gene. Each sample was done in triplicate, and the relative amount of mRNA was normalized to the 18*S* RNA levels in each sample. Normalized expression values (specific gene expression levels/18*S* expression levels) are represented. Values in parentheses indicate the fold induction of mRNA levels relative to the untreated sample for each gene. (**C**) HeLa cells transduced with LV-siGFP or LV-siELKS were transfected with an HIV–NF-κB luciferase reporter construct, untreated or treated with TNF-α or IL-1 (25 ng/mL) for 4 hours, and assessed for NF-κB–dependent transactivation of the luciferase reporter gene. (**D**) HeLa cells transduced with LV-siGFP or LV-siELKS were treated with TNF-α (50 ng/mL) for the indicated time points. Staining of nuclear chromatin with Hoechst 33342 (30 min, 37°C) was used for identification of morphologic changes by fluorescence microscopy (top). Enlarged inset (bottom, right) is representative of a nucleus scored for apoptosis (inset, right) as compared with a normal nucleus (inset, left). Each percentage scoring for apoptotic nuclei (marked by arrowheads) was established by counting 400 cells for each condition. Quantification of the extent of apoptosis is shown (bottom). (**E**) Equal amounts of total lysates from LV-siGFP or LV-siELKS cells treated with TNF-α (50 ng/ml, 6 hours) were resolved on SDS-PAGE and immunoblotted with the indicated antibodies.

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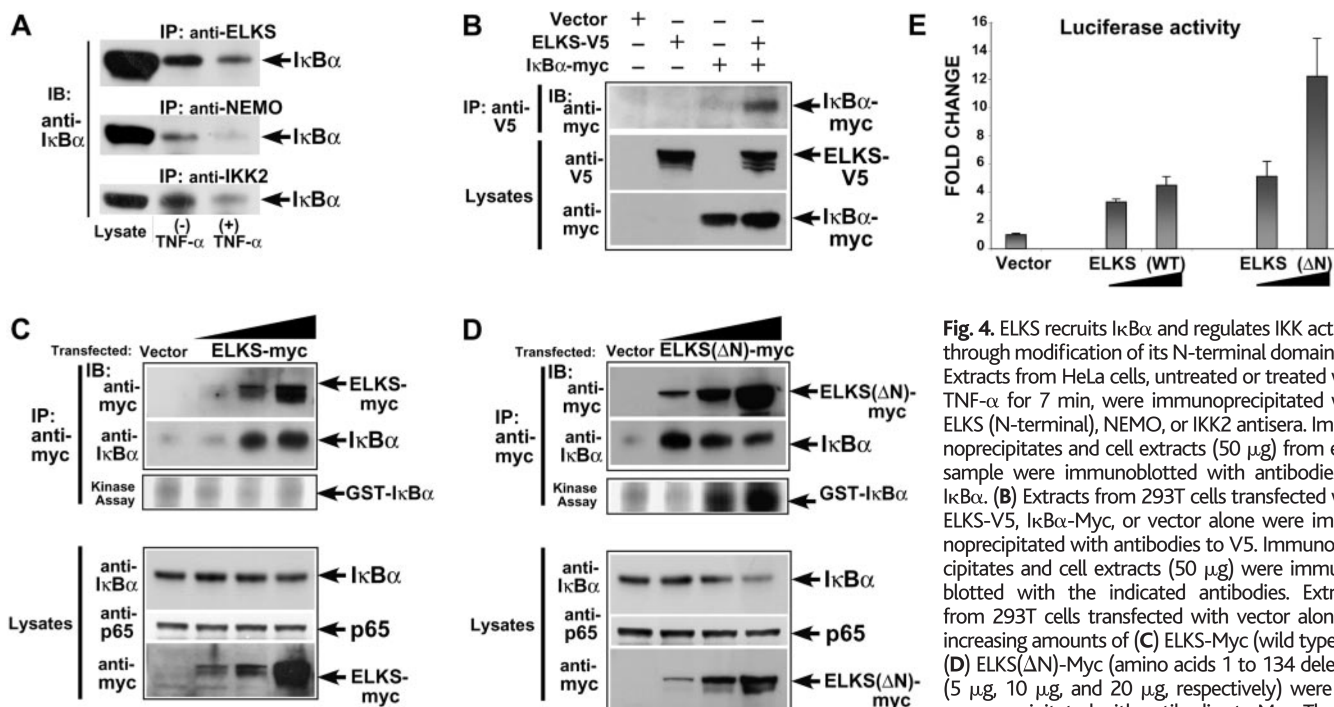


**Fig. 4.** ELKS recruits IκBα and regulates IKK activity through modification of its N-terminal domain. (**A**) Extracts from HeLa cells, untreated or treated with TNF-α for 7 min, were immunoprecipitated with ELKS (N-terminal), NEMO, or IKK2 antisera. Immunoprecipitates and cell extracts (50 μg) from each sample were immunoblotted with antibodies to IκBα. (**B**) Extracts from 293T cells transfected with ELKS-V5, IκBα-Myc, or vector alone were immunoprecipitated with antibodies to V5. Immunoprecipitates and cell extracts (50 μg) were immunoblotted with the indicated antibodies. Extracts from 293T cells transfected with vector alone or increasing amounts of (**C**) ELKS-Myc (wild type), or (**D**) ELKS(ΔN)-Myc (amino acids 1 to 134 deleted) (5 μg, 10 μg, and 20 μg, respectively) were immunoprecipitated with antibodies to Myc. The immunoprecipitates were split into two equal parts. Half the sample was resolved on SDS-PAGE and immunoblotted with antibodies to IκBα and Myc. The other half of the sample was assessed for IKK activity by an in vitro immune complex kinase assay with GST-IκBα(1–54) protein as substrate. (bottom) Cell extracts (50 μg) from each treated sample were resolved on SDS-PAGE and immunoblotted with the indicated antibodies. (**E**) HeLa cells were transfected with vector alone (3 μg), ELKS-Myc [wild type (WT)] (0.3 μg and 3.0 μg, respectively), and ELKS(ΔN)-Myc (0.3 μg and 3.0 μg, respectively) in combination with an HIV–NF-κB luciferase reporter vector, and assessed for NF-κB–dependent transactivation 48 hours after transfection.

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Materials and Methods
Figs. S1 and S2
References

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# Computational Design of a Biologically Active Enzyme

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**Rational design of enzymes is a stringent test of our understanding of protein chemistry and has numerous potential applications. Here, we present and experimentally validate the computational design of enzyme activity in proteins of known structure. We have predicted mutations that introduce triose phosphate isomerase activity into ribose-binding protein, a receptor that normally lacks enzyme activity. The resulting designs contain 18 to 22 mutations, exhibit $10^5$- to $10^6$-fold rate enhancements over the uncatalyzed reaction, and are biologically active, in that they support the growth of *Escherichia coli* under gluconeogenic conditions. The inherent generality of the design method suggests that many enzymes can be designed by this approach.**

Enzymes are among the most proficient catalysts known (*1*), and they catalyze a wide variety of reactions in aqueous solutions under ambient conditions with exquisite selectivity and stereospecificity (*2*, *3*). The rational design of enzymes has tremendous practical potential for developing novel synthetic biochemical pathways (*4*, *5*), but presents a formidable challenge and is one of the most stringent tests for understanding protein chemistry. Here, we present structure-based computational design techniques (*6*, *7*) that predict mutations for the construction of catalytically active sites in proteins of known structure. Using these methods, we converted ribose-binding protein (*8*) into analogs (NovoTims) of the glycolytic enzyme triose phosphate isomerase (*9*). Several NovoTims exhibit rate enhancements of about $10^5$ to $10^6$ and are biologically active, as seen in their support of the growth of *Escherichia coli* under gluconeogenic conditions.

Triose phosphate isomerase (TIM) is an essential component of the Embden-Meyerhof pathway (*10*), interconverting dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GAP) (Fig. 1A). In glycolysis, TIM channels these two triose phosphate products of aldolase into pyruvate; in gluconeogenesis,