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An RNA Ligase Essential for RNA Editing and Survival of the Bloodstream Form of *Trypanosoma brucei*

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RNA editing in trypanosomes occurs by a series of enzymatic steps that are catalyzed by a macromolecular complex. The TbMP52 protein is shown to be a component of this complex, to have RNA ligase activity, and to be one of two adenylatable proteins in the complex. Regulated repression of *TbMP52* blocks editing, which shows that it is a functional component of the editing complex. This repression is lethal in bloodforms of the parasite, indicating that editing is essential in the mammalian stage of the life cycle. The editing complex, which is present in all kinetoplastid parasites, may thus be a chemotherapeutic target.

RNA editing in kinetoplastid protozoa produces mature mitochondrial (mt) mRNAs by a series of catalytic steps that insert and delete the number of uridylates (Us) specified by guide RNAs (gRNAs). The pre-mRNA undergoes endonucleolytic cleavage, U addition or removal, and ligation. The gRNAs duplex with the pre-mRNAs, and these RNAs associate with the multiprotein complex that catalyzes the editing (*1–3*). We recently purified the editing complex of *Trypanosoma brucei* and identified several candidate genes for its components (*4*). Monoclonal antibody (mAb) P3C1-G2, which is specific for recombinant and native protein from one of these genes, *TbMP52*, immunoprecipitates in vitro editing activity, indicating that the TbMP52 protein is a component of the editing complex (*4*). We report here that *TbMP52* encodes an RNA ligase and that down-regulation of its expression in vivo stops the production of edited mRNA and results in the death of the bloodforms of the parasite.

The predicted *T. brucei* TbMP52 protein sequence has high homology to a putative ortholog in *Leishmania major* and to TbMP48, a putative paralog of TbMP52 in *T. brucei* that is also present in the purified editing complex (*4*). Although no homologs with known function could be identified, motif and domain searches (*5*) identified ligase signature boxes (*6*) in both TbMP52 and TbMP48 (Fig. 1). This includes the KXXG (*7*) box (region I, Fig. 1), the lysine of which becomes adenylated in ligases during ligation (*8*). These findings suggested that *TbMP52* and *TbMP48* encode the larger and the smaller adenylatable protein, respectively, that are present in purified RNA editing complexes and have been suggested to represent editing ligases (*9–11*). In vitro–transcribed and –translated recombinant TbMP52 protein (rTbMP52) ligated 71% of a synthetic RNA substrate (Fig. 2, A and B). rTbMP52 also autoadenylated upon incubation with [^{32}P]α–adenosine triphosphate (α-ATP) (Fig. 2C). mAb P3C1-G2, which is specific for TbMP52, immunoprecipitated the larger of the two adenylatable proteins from native complexes after dissociation into individual proteins (Fig. 2D). TbMP48 also contains the KXXG box, and its predicted size matches that of the smaller adenylatable protein (*9*, *11*). Thus, *TbMP48* is likely to encode this second adenylatable component of the complex, which may also be an RNA ligase.

The in vivo function of *TbMP52* was investigated by targeted gene replacement of both endogenous alleles and insertion of a *TbMP52* allele that was under the control of a tetracycline (tet) inducible promoter, leading to mutant clone 1B3 (*12–14*). Western analysis showed that *TbMP52* is conditionally expressed in clone 1B3 (Fig. 3A). The amount of TbMP52 protein was substantially reduced in non-induced (no tet) cells as compared to induced cells. Loss of TbMP52 also led to a substantial reduction in complexes containing adenylatable proteins that can be immunoprecipitated (Fig. 3B). mAb P1H3-D7, which is specific for a 69-kD component of the editing complex, immunoprecipitates active editing complexes (*15*) and complexes that contained both adenylatable proteins from wild-type and induced 1B3 cells. TbMP52 and TbMP48 were reduced by 91.3 and 42.8% in the precipitates of non-induced 1B3 cells, respectively. Thus, there appear to be fewer editing complexes in cells with reduced TbMP52, which indicates that this protein is an integral component of the editing complex.

TbMP52 expression was found to be essential for proliferation of *T. brucei*. Clone 1B3 grew continuously in vitro in the presence of tet, with a generation time indistinguishable from that of the wild type (Fig. 3C, solid squares). However, repression of *TbMP52* expression in the absence of tet resulted in cessation of growth after about 70 hours (Fig. 3C, open squares). Over 95% of the population died within 48 hours, and many lysed cells were evident. Reintroduction of tet 21 hours after cessation of growth (91 total hours, arrow in Fig. 3C) prevented the population decline, and growth resumed at the normal rate approximately 90 hours later (181 total hours), indicating that the cell death and population

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decline were due to repression of *TbMP52* gene expression. Some cells survived in the absence of tet, but ultimately died (Fig. 3C) or resumed growth at a normal rate. This survival may be due to mutations in the *PARP* promoter or in the tet repressor gene, or to compensatory changes that bypass the role of TbMP52 in bloodstream-form parasites.

Expression of TbMP52 is essential for the growth of bloodforms both in culture and in mice (Table 1). In the absence of doxycycline (dox, non-induced *TbMP52*), parasites of clone 1B3 initially grew but then declined to an undetectable level ~105 hours after infection. However, in the presence of dox, the parasites continued to multiply and the mice died 105 to 128 hours after infection, depending on the initial level of infection. Mice died within 105 hours of infection with the parental parasite clone 1-5, which contains one endogenous allele in addition to the regulated ectopic copy (*12*), regardless of the presence or absence of dox.

Inactivation of the expression of the sole ectopic allele of *TbMP52* in clone 1B3 resulted in the loss of detectable fully edited mRNA within 46 hours (Fig. 4). RNA was isolated at different times from cells grown in the presence or absence of tet (numbers 1 to 5, Fig. 4A). Unedited, partially edited, and fully edited mRNAs for ATPase subunit 6 (*A6*), reduced nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 7 (*ND7*), and ribosomal protein small subunit 12 (*RPS12*) RNAs were evident upon reverse transcription polymerase chain reaction (RT-PCR) analysis of RNA from cells grown in the presence of tet (Fig. 4, B and C). However, RT-PCR products from fully edited RNA were absent, and a smaller spectrum was produced within 46 hours after the withdrawal of tet. The smaller products may represent partially edited RNA that remained after editing ceased and/or aberrantly edited RNA. However, this overall decrease in RNA size suggests that editing is not processive for the entire mRNA molecule. The RT-PCR product representing *ND4*, which does not get edited, did not decrease during the time course, indicating that mt transcription is still functioning and that the observed changes are specific and not the result of a general RNA degradation in the non-induced cultures. The normal growth for ~48 hours after the loss of fully edited RNA suggests that the turnover rate of the protein products of these RNAs is slow enough to provide an amount sufficient to sustain growth for at least four cell divisions. Poison primer extension analysis of the ND7 mRNA showed that less than 2% of edited transcripts remained 75 hours after removal of tet, as compared to ~55% in induced cells (*16*). Overall these results show that TbMP52 is an RNA ligase with a critical role in RNA editing. Consequently, TbMP52 is named TbREL1 (for *T. brucei* RNA editing ligase 1), the *L. major* homolog is named LmREL1, and TbREL2 is reserved for TbMP48. These proteins have a higher sequence homology to DNA ligases than to RNA ligases (Fig. 1, conserved boxes III and IIIa). This may reflect the double-stranded substrates of the editing ligases and perhaps an evolutionary origin in common with DNA ligases, some of which have been shown to ligate RNA molecules that are bridged by a DNA (*17*).

TbMP52 is essential for RNA editing. Other candidate editing complex proteins have been identified, but, with the excep-

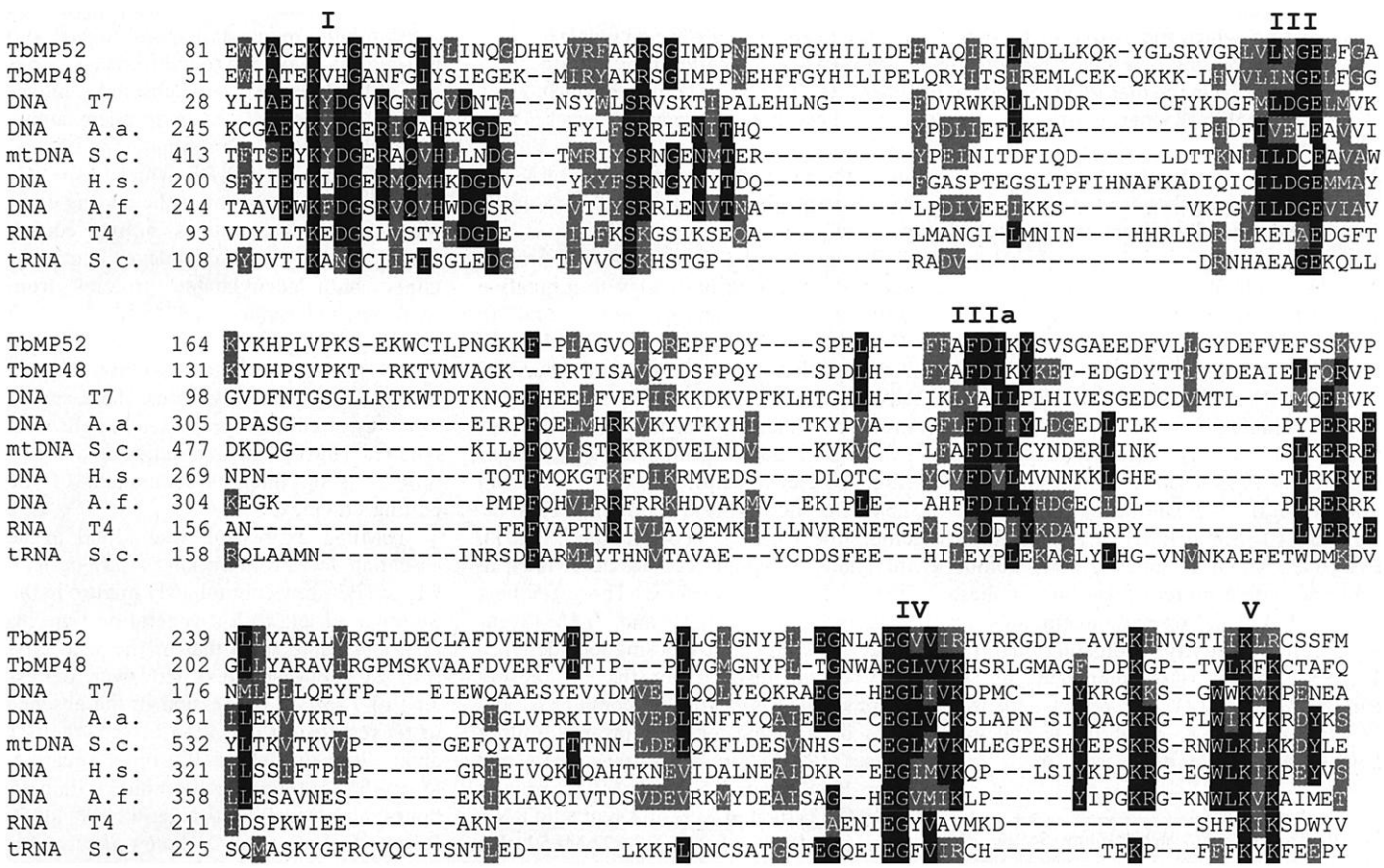

Fig. 1. Alignment of TbMP52 and TbMP48 with DNA and RNA ligases (*7*). Identities are in black and similarities are in gray; box designations (I through V) are according to (*6*). Abbreviations (with GenBank accession numbers in parentheses) are as follows: TbMP52 (AAG27062); TbMP48 (AAG27063); DNA T7, DNA ligase bacteriophage T7 (3114525); DNA A.a., DNA ligase *Aquifex aeolicus* (7447282); mtDNA S.c., mitochondrial DNA ligase *Saccharomyces cerevisiae* (2506361); DNA H.s., DNA ligase IV *Homo sapiens* (1706482); DNA A.f., DNA ligase *Archaeoglobus fulgidus* (6014986); RNA T4, RNA ligase bacteriophage T4 (133093); tRNA S.c., tRNA ligase *S. cerevisiae* (136250).

tion of the RNA helicase mHel61p, it has not yet been demonstrated that they have a role in editing. The role of mHel61p in editing is uncertain because null mutants still edit, but with reduced activity, and they grow slowly (*18*). TbMP52 and TbMP48 do not appear to be functionally redundant because TbMP48 does not compensate for the loss of TbMP52, at least in bloodforms. The two ligases might each have a different function (for example, in deletion versus insertion editing), or one ligase might function more efficiently in bloodforms and the other in insect forms.

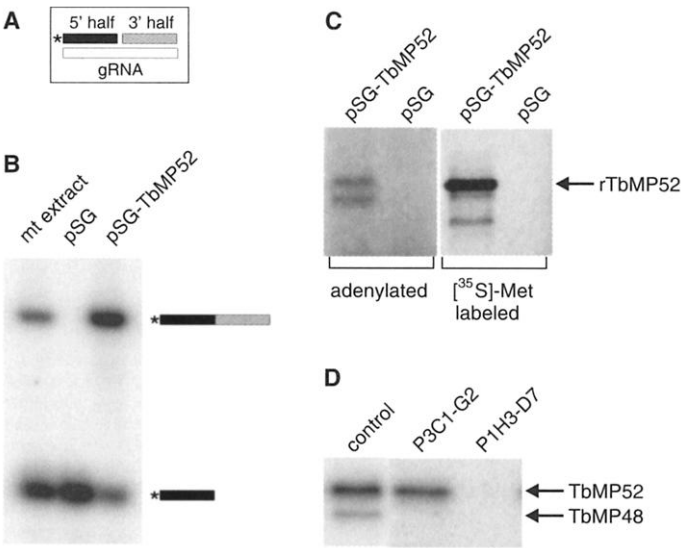

Fig. 2. TbMP52 is an RNA ligase and is the larger adenylatable protein. (**A**) Diagram of the no-gap ligation substrate (*25*). The asterisk indicates the 5′ label. (**B**) RNA ligase assay of immunoprecipitated translation products from pSG-TbMP52 and pSG (*24*) and of mt extract (*4*). (**C**) Autoadenylation assay (*9*) of rTbMP52 and controls as in (B). [^{35}S]methionine(Met)–labeled rTbMP52 served as a size marker. The smaller adenylated band appears to be a degradation product of rTbMP52. (**D**) mAb P3C1, which is specific for TbMP52, immunoprecipitates the upper adenylatable band. Editing complexes (*4*) were autoadenylated with [^{32}P]α-ATP and loaded directly (control) or after dissociation (in 2% SDS and 1M NaCl at 100°C for 5 min) and immunoprecipitation (after dilution to 0.1% SDS), using mAbs P3C1-G2 or P1H3-D7, which are specific for a 69-kD component of the editing complex (*15*).

We have shown that *TbMP52* is essential for the growth of bloodform *T. brucei* even though RNA editing was believed not to be essential for this life-cycle stage. The mtDNA encodes components of the oxidative phosphorylation system (subunits of NADH dehydrogenase, cytochrome oxidase, and ATP synthase, as well as apocytochrome b), and most of the respective transcripts undergo RNA editing. Bloodforms lack cytochromes and rely on glycolysis for ATP production, whereas insect forms produce most ATP by cytochrome-mediated oxidative phosphorylation (*19*). The ability of dyskinetoplastic (dk) mutants (which have gross mtDNA deletions) to grow as bloodforms but not as insect forms (*20*) is consistent with this view. However, bloodforms have rotenone-sensitive NADH dehydrogenase activity (*21*) and preferentially edit mRNAs for this complex (*22*, *23*). Thus, the loss of editing of NADH dehydrogenase subunits [such as ND7 (Fig. 4C)] would be lethal to cells that require this activity. Hence, this indicates that RNA editing is essential for normal bloodform *T. brucei*. The dk mutants may have compensatory changes that bypass the need for RNA editing. We cannot exclude the possibility that TbMP52 may be a multifunctional protein, perhaps with additional roles in RNA processing. An essential role for RNA editing in bloodforms suggests that RNA editing may provide chemother-

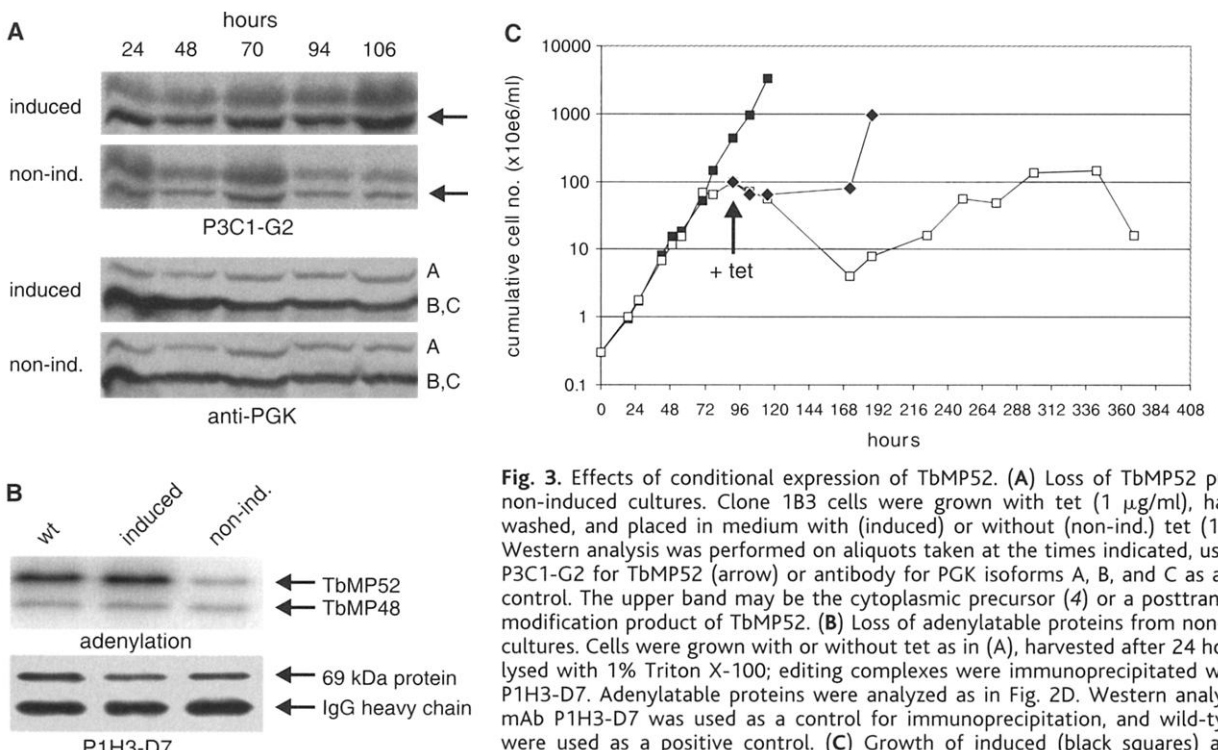

Fig. 3. Effects of conditional expression of TbMP52. (**A**) Loss of TbMP52 protein in non-induced cultures. Clone 1B3 cells were grown with tet (1 μg/ml), harvested, washed, and placed in medium with (induced) or without (non-ind.) tet (1 μg/ml). Western analysis was performed on aliquots taken at the times indicated, using mAb P3C1-G2 for TbMP52 (arrow) or antibody for PGK isoforms A, B, and C as a loading control. The upper band may be the cytoplasmic precursor (*4*) or a posttranslational modification product of TbMP52. (**B**) Loss of adenylatable proteins from non-induced cultures. Cells were grown with or without tet as in (A), harvested after 24 hours, and lysed with 1% Triton X-100; editing complexes were immunoprecipitated with mAb P1H3-D7. Adenylatable proteins were analyzed as in Fig. 2D. Western analysis with mAb P1H3-D7 was used as a control for immunoprecipitation, and wild-type cells were used as a positive control. (**C**) Growth of induced (black squares) and non-induced (white squares) cultures. Cumulative cell numbers reflect normalization for dilution during cultivation. The non-induced culture was split after 91 hours (arrow), and tet (1 μg/ml) was added to one culture (black diamonds). The results were reproducible over the first 170 hours, but there was variation in the subsequent fate of the non-induced cells, as discussed.

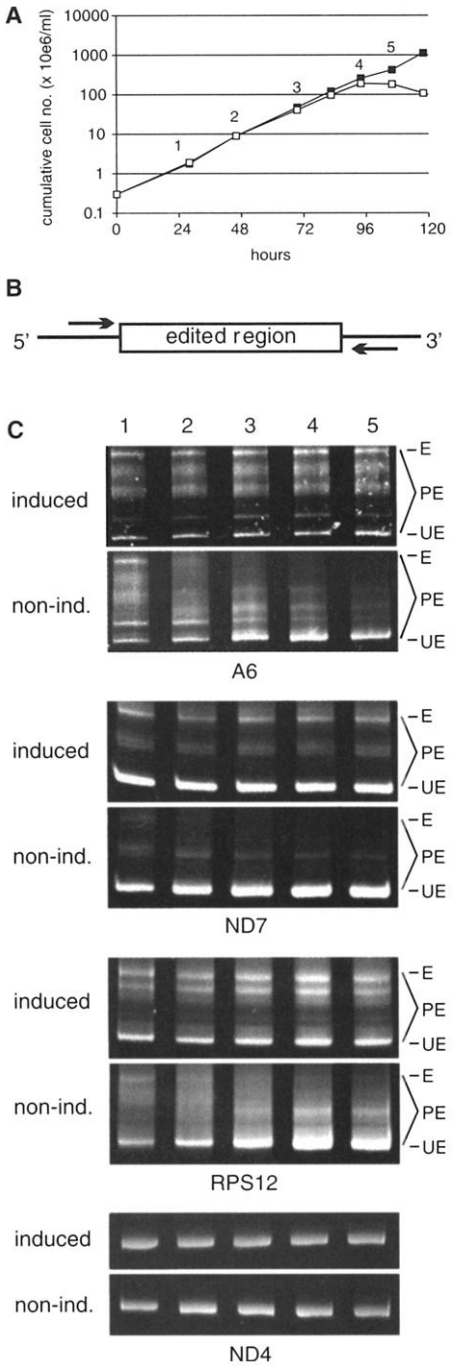

Fig. 4. Loss of TbMP52 blocks RNA editing. (**A**) Growth of induced (black squares) and non-induced (white squares) parasites. Total RNA was prepared from samples taken at the time points indicated (*16*). (**B**) Diagram showing RT-PCR primer locations relative to the edited region. (**C**) Polyacrylamide gel analysis of RT-PCR products from RNAs that are normally edited in bloodforms (A6, ND7, and RPS12) and ND4 RNA, which does not get edited (*16*). Fully edited (E), partially edited (PE), and unedited (UE) molecules were identified by cloning and sequencing.

Table 1. Growth in mice of *T. brucei* with induced or non-induced TbMP52. C57 BL/6 mice were infected by intraperitoneal inoculation with 10^5 cultured bloodstream-form *T. brucei* clones 1B3 or 1-5 (the parental clone with one endogenous allele plus the ectopic allele). Dox (200 μg/ml) and/or 5% sucrose was added to the drinking water beginning 1 week before infection for induction. Parasites were counted with a hemocytometer.

Genotype	dox	Parasites ($\times 10^6$ per milliliter of blood)			
		66 hours	81 hours	105 hours	128 hours
1B3	−	3	1	0	0
	−	16	41	0	0
	+	10	232	*	
	+	0	4	78	955*
1-5	−	26	395	*	
	−	14	239	*	
	+	4	266	*	

*These mice died of the infection or were killed because of high parasitemia.

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Defective Lymphotoxin-β Receptor–Induced NF-κB Transcriptional Activity in NIK-Deficient Mice

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The role of NF-κB–inducing kinase (NIK) in cytokine signaling remains controversial. To identify the physiologic functions of NIK, we disrupted the NIK locus by gene targeting. Although NIK$^{-/-}$ mice displayed abnormalities in both lymphoid tissue development and antibody responses, NIK$^{-/-}$ cells manifested normal NF-κB DNA binding activity when treated with a variety of cytokines, including tumor necrosis factor (TNF), interleukin-1 (IL-1), and lymphotoxin-β (LTβ). However, NIK was selectively required for gene transcription induced through ligation of LTβ receptor but not TNF receptors. These results reveal that NIK regulates the transcriptional activity of NF-κB in a receptor-restricted manner.

apeutic targets for kinetoplastid pathogens, which employ RNA editing (such as African and American trypanosomes and *Leishmania*).

The transcription factor NF-κB is activated by a variety of cell surface receptors (*1*). Although different receptors often use distinct combinations of intracellular proteins to initiate NF-κB activation, the signals converge downstream into a common pathway