Doctorate Program in Molecular Oncology and Endocrinology Doctorate School in Molecular Medicine

XXI Cycle 2005-2008
Coordinator: Professor Giancarlo Vecchio

"Regulation of the NF-κB alternative pathway: implication for B cell transformation"

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2. Iannetti A, Pacifico F, Acquaviva R, **Lavorgna A**, Crescenzi E, Vascotto C, Tell G, Salzano AM, Scaloni A, Vuttariello E, Chiappetta G, Formisano S, Leonardi A. *The neutrophil gelatinase-associated lipocalin (NGAL), a NF-{kappa}B-regulated gene, is a survival factor for thyroid neoplastic cells*. **Proc Natl Acad Sci U S A.** 2008 Sep 3

3. Mauro C, Giaccone G, Piscosquito G**, Lavorgna A**, Nigro M, Di Fede G, Leonardi A, Coppola C, Formisano S, Tagliavini F, Cotrufo R, Puoti G. *A novel insertional mutation in the prion protein gene: clinical and bio-molecular findings.* **J Neurol Neurosurg Psychiatry.** 2008 Dec;79(12):1395-8

ABSTRACT

The NF-κB2 gene is recurrently mutated and over-expressed in human lymphoid malignancies. However, a casual relationship between NF-κB2 mutation and lymphomagenesis has not been established. It is also unclear how the mutation may lead to lymphoid malignancies. Recent studies suggest that nuclear factor κB inducing kinase (NIK) is suppressed through constitutive proteasome-mediated degradation regulated by TRAF3, thus preventing processing of the NF-κB2 precursor protein p100 to release p52. Here we demonstrate that BAFF Receptor, a member of the TNF Receptor family, interact with TRAF1, a member of TRAF family. This interaction activates NF-κB2 via increased degradation of TRAF3 and stabilization of NIK. Indeed, interference of TRAF1 in lymphoma B cell line downregulates p100 processing and lead to decreased survival of B cells.

BACKGROUND

Introduction

The inducible regulation of gene expression is a central element of normal physiology and is the key to the ability of multicellular organisms to adapt to environmental, mechanical, chemical and microbiological stresses. Owing to its importance in disease, NF-κB has served as a model of cell, tissue and organism level responses that are orchestrated through inducible transcription factors. NF-κB plays its most important and evolutionary conserved role in the immune system, regulating the expression of inducers and effectors at many point in the expensive networks that define responses to pathogens. The reach of NF-κB, however, extends to transcriptional regulation beyond the confines of the immune response, acting broadly to influence gene expression events that impact cell survival, differentiation and proliferation. The diversity of biological roles fulfilled by NF-κB raised several intriguing question about how a limited set of signal transduction molecules regulates signaling to NF-kB in all pathway and, conversely, how discrete inputs create transcriptional responses tailored to particular tissues and organs with the same limited set of regulators.

The basic scheme of NF-κB signaling consists in a series of positive and negative regulatory elements. Inducing stimuli trigger IKK activation leading to phosphorilation, ubiquitination and degradation of IκBs proteins. Released NF-κB dimers are further activated through various posttranslational modifications and translocate to the nucleus where they bind to specific DNA sequences and promote transcription of target genes. In its most basic form, therefore, the pathway consists of receptor and receptor proximal signaling adaptor molecules, the IKK complex, IκB proteins and NF-κB dimers.

The NF-κB family of transcription factors consists of five members, p50, p52, p65 (RelA), c-Rel and RelB, encoded by NFKB1, NFKB2, RELA, REL and RELB, respectively, which share an N-terminal Rel homology domain (RHD) responsible for DNA binding and homo- and heterodimerization (Fig.1). NF-κB dimers bind to κB sites within the promoters/enhancers of target genes, and regulate transcription through the recruitment of coactivators and corepressors. The transcription activation domain (TAD) necessary for the positive regulation of gene expression, is present only in p65, c-Rel and RelB. As they lack TADs, p50 and p52 may repress transcription unless associated with a TAD-containing NF-κB family member, or other proteins capable of coactivator recruitment. There is considerable structural information about NF-κB dimers in both its inactive IκB-bound form and active DNA bound state. Crystal structures of NF-κB dimers bound to κB sites reveal how the immunoglobulin-like domains that comprise the RHD contact DNA. The NH2-terminal Ig-like domain confers selectivity for certain types of κB sites, whereas the hydrophobic residues within the C-terminal domain provide the dimerization interface between NF-κB subunits (Hoffmann et al., 2006). In its inactive state, NF-κB dimers are associated with one of three

typical IκB proteins, IκBα, IκBβ, or IκBε, or the precursor proteins p100 (*NFKB2*) and p105 (*NFKB1*). These IκBs maintain NF-κB dimers in the cytoplasm, and are crucial for signal responsiveness. There are two inducibly expressed, atypical IκB proteins, Bcl-3 (*BCL3*) and IκBζ (*NFKBZ*), that function quite differently in the regulation of NF-κB. Lastly an alternative transcript of the *NFKB1* gene in mouse encodes an IκB molecule, IκBγ, whose biological role remains unclear. All IκB proteins are characterized by the presence of multiple ankyrin repeat domains (Figure 1). The prototypical and most extensively studied member of the family is IκBα. IκBα is rapidly degraded during activation of canonical NF-κB signaling pathway, leading to the release of multiple NF-κB dimers, although the p65:p50 heterodimer is likely the primary target of IκBα. The established model of IκB function posits that IκBα retains NF-κB dimers in the cytoplasm, thereby preventing their nuclear translocation, and subsequent DNA binding; however, the situation is actually more complex. The crystal structure of IκBα bound to the p65/p50 heterodimer reveals that the IκBα protein masks only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains exposed. The exposed NLS of p50 coupled with nuclear export sequences (NES) in IκBα and p65 leads to constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm, despite steadystate localization that appears almost exclusively cytosolic (Ghosh and Karin, 2002). Degradation of IκBα drastically alters the dynamic balance between cytosolic and nuclear localization signals to favor nuclear localization of NF-κB.

The noncanonical or alternative NF-κB pathway, proceeds through proteasomal processing, rather than degradation, of p100 to p52, thereby liberating p52 containing NF-κB dimers, that drive a transcriptional response that is distinct from that induced by the canonical, IκBα-regulated pathway. In part because IκBα degradation and p100 processing regulate different populations of NF-κB dimers, canonical and noncanonical NF-κB pathways regulate distinct sets of target genes. Degradation of IκB is a rapidly induced signaling event that is initiated upon specific phosphorylation of these molecules by activated IKK. The IKK complex contains two highly homologous kinase subunits, IKKα/IKK1 and IKKβ/IKK2 and a regulatory subunit NEMO (NF-κB essential modulator)/IKKγ (Hacker and Karin, 2006). Although they are generally found in a heteromeric kinase complex, IKKα and IKKβ are somewhat selectively required for specific NF-κB signaling pathways. In most canonical NF-κB signaling, downstream of TNFR1, IKKβ is both necessary and sufficient for phosphorylation of IκBα on Ser32 and Ser36, and of IκBβ on Ser19 and Ser23. While not generally required for IκBα phosphorylation and degradation in canonical signaling pathways, IKKα can mediate IκBα phosphorylation and appears to play critical role in canonical NF-κB-dependent transcriptional responses. The noncanonical pathway, conversely, depends only on the IKKα subunit, which functions by phosphorylating p100 and causing its inducible processing to p52. The noncanonical pathway is activated by a subset of TNFR superfamily members, while the canonical pathway is activated by a broader and overlapping array of receptors. Phosphorylation of the conserved serine residues (DS*GXXS*) in IκB proteins results in their K48-linked

polyubiquitination by βTrCP containing Skp1-Culin-Roc1/Rbx1/Hrt-1-F-box (SCF) E3 ubiquitin ligase complexes (SCFβTrCP) coordinately with the E2 UbcH5 (Perkins, 2006). The released NF-κB dimers bind promoter and enhancer regions containing κB consensus sequences 5′ GGGRNWYYCC 3′ (N—any base; R—purine; W—adenine or thymine; and Y—pyrimidine) (Hoffmann et al., 2006). The degenerate nature of the κB site sequence, which shows far greater sequence variability than the consensus sequence given here, combined with the varied binding preferences of NF-κB dimers yields the large list of NF-κB-regulated genes (Gilmore, 2008). Transcription of target genes is further regulated through posttranslational modifications of NF-κB that affect the ability of NF-κB dimers to interact with transcriptional coactivators. NF-κB-dependent transcription of IκB proteins as well as additional mechanisms targeting DNA-bound NF-κB dimers terminate the response.

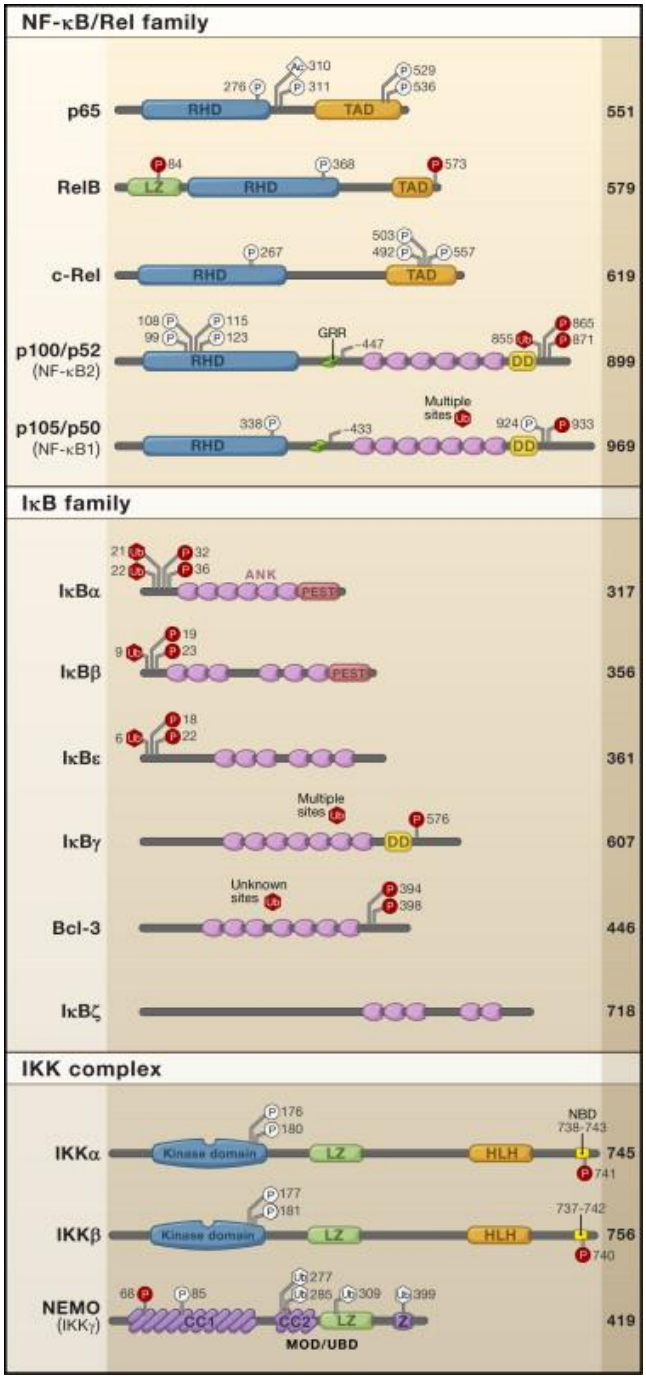


Figure 1: The NF-κB, IκB, and IKK Protein Families
Members of the NF-κB, IκB, and IKK proteins are shown. The number of amino acids in each human protein is indicated on the right. Posttranslational modifications that influence IKK activity or transcriptional activation are indicated with P, U, or Ac for phosphorylation, ubiquitination, or acetylation, respectively. Inhibitory events and phosphorylation and ubiquitination sites on p100, p105, and IκB proteins that mediate proteasomal degradation are indicated with red Ps and Us, respectively. RHD, Rel homology domain; TAD, transactivation domain; LZ, leucine zipper

domain; GRR, glycine-rich region; HLH, helix-loop-helix domain; Z, zinc finger domain; CC1/2, coiled-coil domains; NBD, NEMO-binding domain; MOD/ UBD, minimal oligomerization domain and ubiquitin-binding domain; and DD, death domain.
Matthew S. Hayden and Sankar Ghosh. Review. Cell 2008

Signaling to IKK

A remarkable diversity of stimuli lead to activation of NF-κB. These include both endogenous and exogenous ligands, as well as a plethora of physical and chemical stresses (Gilmore, 2008). There has been great progress in identifying the protein components of pathways that culminate in IKK activation, and many of the remaining gaps in our knowledge are being rapidly filled in. However, while the identities of these molecules provide critical clues, much remains to be done to understand the mechanisms of IKK activation.
Strikingly, work in numerous signaling pathways leading to NF-κB has demonstrated that many of the signaling intermediates, especially those just upstream of the IKK complex, are shared. Signaling to NF-κB proceeds through intracellular adaptor proteins that provide modularity to NF-κB activation pathways and allows their incorporation into various receptor induced signaling events. Thus diverse signaling pathways can utilize several shared components for both activating and inhibitory pathways. In particular, RIP and TRAF families of proteins play similar roles in most pathways that lead to IKK activation (Figure 2). Although there are exceptions, some of which are noted below, it appears that both canonical and noncanonical pathways utilize TRAF family members for activation, while only canonical, NEMO-dependent signaling to typical IκBs additionally requires RIP proteins. One striking area of growth in the field of NF-κB signaling has been in the characterization of the role of K63-linked, or regulatory, ubiquitination. This area of research began with the biochemical characterization of TRAF6 as an E3 ligase that with the E2 ligase Ubc13/Uev1A could catalyze the formation of regulatory ubiquitin chains and induce IKK activation in vitro (Deng et al., 2000). Subsequently, several signaling components have been shown to be modified by K63-linked ubiquitin moieties following stimulation (Chen et al., 2006). While there are numerous reports demonstrating K63 ubiquitination of various signaling proteins, what continues to be lacking is an understanding of how regulatory ubiquitination functions during signaling. The delayed kinetics of regulatory ubiquitination and the observation that it often targets a very small fraction of any given protein, even within signaling complexes, suggest that K63 ubiquitination may be a consequence of certain adapters with E3 ligase activity aggregating with other proteins during NF-κB signaling. Even for particularly robustly and rapidly ubiquitinated adapters such as IRAK1 and MALT1, it is unclear whether K63-linked ubiquitination occurs before IKK activation and IκBα degradation (Oeckinghaus et al., 2007; Windheim et al., 2008). Because K63-linked ubiquitination has not been clearly shown to precede IKK activation, it remains to be determined whether these

events are intrinsic to the act of signaling, or necessary for the competence of the signaling pathways in which they occur. One piece of evidence that strongly suggests that K63 ubiquitination does play an active role in signaling is the existence of several deubiquitinases (DUBs), most notably A20, that provide negative feedback in NF-κB signaling pathways (Chen et al., 2006, Mauro et al., 2006). More genetic evidence and mechanistic insight are still needed for the centrality of regulatory ubiquitination in NF-κB signaling to be unequivocally accepted.

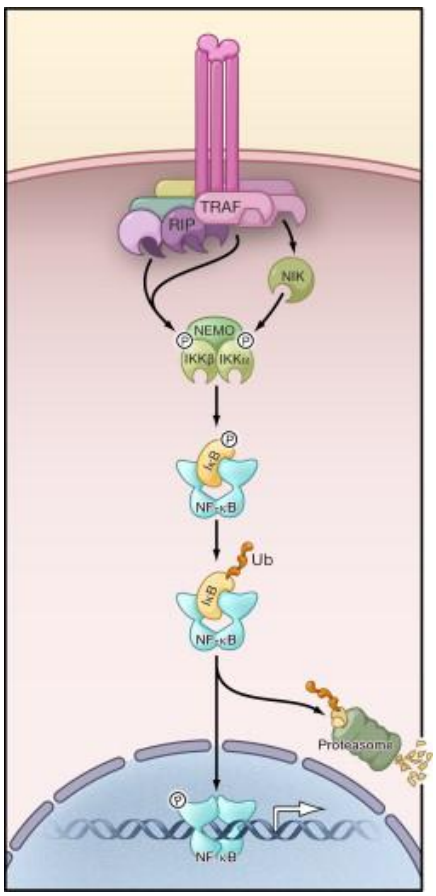


Figure 2: NF-κB Signaling Pathways
Following receptor ligation and recruitment of receptor proximal adaptor proteins, signaling to IKK proceeds through TRAF/RIP complexes, generally in conjunction with TAK1, leading to canonical NF- κB signaling, or through TRAFs and NIK leading to the noncanonical NF-κB pathway. IKK activation results in IκB phosphorylation and degradation in the canonical pathway or p100 processing to p52 in the noncanonical pathway. Phosphorylated NF-κB dimers bind to κB DNA elements and induce transcription of target genes. Matthew S. Hayden and Sankar Ghosh. Review. Cell 2008

TRAFs - Adapters in Most NF-κB Pathways

TRAFs are key intermediates in nearly all NF-κB signaling pathways; the DNA-damage response appears to be the only notable exception (Hacker and Karin, 2006; Scheidereit, 2006). There are seven TRAF proteins that share a C-terminal TRAF domain, consisting of a coiled-coil domain that mediates both homo- and heterotypic protein-protein interactions. In addition, TRAFs 2–7 have N-terminal RING finger domains that may function as E3 ubiquitin ligases by catalyzing the transfer of ubiquitin to target proteins a function that has been demonstrated most clearly for TRAFs 2 and 6. Among the TRAF proteins, TRAF2, TRAF5, and TRAF6 have been most extensively characterized as positive regulators of signaling to NF-κB. Research focused on the role of K63-linked ubiquitination in NF-κB signaling over the past 7 years has established an important role for TRAF E3 ligase activity in the activation of the IKK complex leading to both canonical and noncanonical NF-κB pathways and demonstrated the existence of K63 ubiquitination of multiple pathway components (Chen et al., 2006). In addition to NF-κB, TRAF proteins are necessary in several other pathways, such as AP-1, and therefore serve as branch points downstream of multiple receptors. The highly studied TNFR1 and Toll/IL-1R signaling pathways, have provided the clearest evidence of the function of TRAF proteins in IKK activation (Hacker and Karin, 2006; Hayden et al., 2006). Following binding of TNFα, TRAF2 is recruited to TNFR1 through its interaction with TRADD (Hsu et al., 1996). However, despite deficiencies in AP-1 activation, TRAF2-deficient cells have relatively intact TNF signaling to NF-κB (Yeh et al., 1997). TRAF5 was also shown to interact with the TNFR1 signaling complex, yet TRAF5 knockouts also exhibit normal NF-κB activation by TNF; TRAF2/5 double knockout cells, however, are defective in IKK activation (Nakano et al., 1999; Tada et al., 2001; Yeh et al., 1997). While the E3 ligase activity of TRAF2 is thought to be required for IKK activation there are several caveats to this assumption. First, deletion of the RING finger domain abrogates the ability of TRAF2 to recruit IKK to the receptor complex, making it difficult to assess the importance of TRAF2 E3-ligase activity independent of adaptor function in NF-κB activation (Devin et al., 2000). Second, knockdown of the K63-specific E2 ligase Ubc13 blocks TRAF2 autoubiquitination, but not activation of NF-κB, while *ubc13*−/− macrophages show a similar lack of effect on NF-κB activation (Habelhah et al., 2004; Yamamoto et al., 2006a). Intriguingly, another group did observe partial defects in TNF signaling to NF-κB in *ubc13*+/− heterozygous macrophages and splenocytes (Fukushima et al., 2007). Therefore, in TNFR1 signaling the function of TRAF2 ubiquitin ligase activity remains to be more definitively established. It is, however, safe to conclude that TRAF2 and TRAF5 are together required for NF-κB activation by TNFR1.

In Toll/IL-1 signaling TRAF6 is recruited to the receptor complex, and is necessary for MyD88-dependent activation of NF-κB by IL-1, and ligands of TLR4 (Hacker and Karin, 2006; Hayden et al., 2006). However, like TRAF2, the importance of the E3-ligase activity of TRAF6 remains controversial. Reconstitution of TRAF6-deficient cells with a TRAF6 mutant lacking the

signature motif of E3 RING-finger ligases—that is, the RING finger itself—completely restored IL-1-induced activation of NF-κB but not activation of JNK (Kobayashi et al., 2001). More recently, however, it has been shown that a ring finger point mutation of TRAF6 is unable to restore NF-κB activation in TRAF6 knockout cells (Lamothe et al., 2007). Therefore, the role of the TRAF6 E3 activity is yet to be definitively established. Deletion of Ubc13, likewise, yields conflicting results. Ubc13 knockouts failed to show significant defects in TRAF6-mediated activation of NF-κB downstream of LPS, IL-1, CD40, or BAFF despite impaired MAPK activation (Yamamoto et al., 2006a). Heterozygous splenocytes and macrophages, however, showed a mild defect in LPS-induced IκBα degradation, less severe than the loss of p38 phosphorylation, and in these mice BCR signaling to NF-κB appeared normal (Fukushima et al., 2007).
In T cell receptor signaling, conditional ablation of Ubc13 resulted in a partial, but significant, reduction in NF-κB activation, although activation of JNK and TAK1 was more severely impaired (Yamamoto et al., 2006b). Therefore, it is probably too early to draw a definitive conclusion about the general role of TRAF/Ubc13-mediated ubiquitination in IKK activation. In addition to TRAF2 and TRAF6, the ubiquitin ligase activity of TRAF3 has been reported to regulate NF-κB signaling pathways.
The noncanonical NF-κB pathway is characterized by processing of p100 to p52, and by its independence from IKKβ and NEMO. Instead the alternative pathway relies on the activation of IKKα by the NF-κB-inducing kinase (NIK) (Hacker and Karin, 2006; Scheidereit, 2006). TRAF3, which interacts with receptors that trigger the alternative pathway (Hauer et al., 2005), also interacts with NIK and it now appears that the activation of NIK is negatively regulated by TRAF3. In the resting state, TRAF3 induces NIK ubiquitination and degradation, but upon stimulation, TRAF3 undergoes signal-dependent degradation, mediated by other TRAF family members, resulting in the accumulation and activation of NIK, and consequent activation of the noncanonical pathway (Liao et al., 2004). Recently this negative role of TRAF3 has been demonstrated genetically by rescuing the lethality of TRAF3-deficient mice by deleting the p100 gene (He et al., 2006). It remains unclear how alternative pathway signaling through degradation of TRAF3 might affect the ability of TRAF3 to fulfill its additional role as a key mediator of TLR-induced type I interferon responses (Hacker et al., 2006; Oganesyan et al., 2006). Thus, TRAF proteins seem to play a crucial role in receptor induced IKK activation in both canonical and noncanonical pathways. It remains unclear whether they act primarily by catalyzing K63-linked ubiquitination, or as adaptor proteins. TRAF proteins may directly recruit the IKK complex through IKKα or IKKβ binding (Devin et al., 2001), although in most signaling pathways additional IKK recruitment mechanisms have been reported. For example, in antigen receptor signaling IKK can be recruited through an interaction with PKC family members while in TNFR1 signaling, IKK may be recruited to the receptor complex through the RIP1 kinase. The key remaining questions are to address the mechanisms by which TRAF proteins contribute to IKK activation, particularly in

conjunction with RIP proteins, and their contributions to NF-κB pathways in which their role has not yet been definitively established.

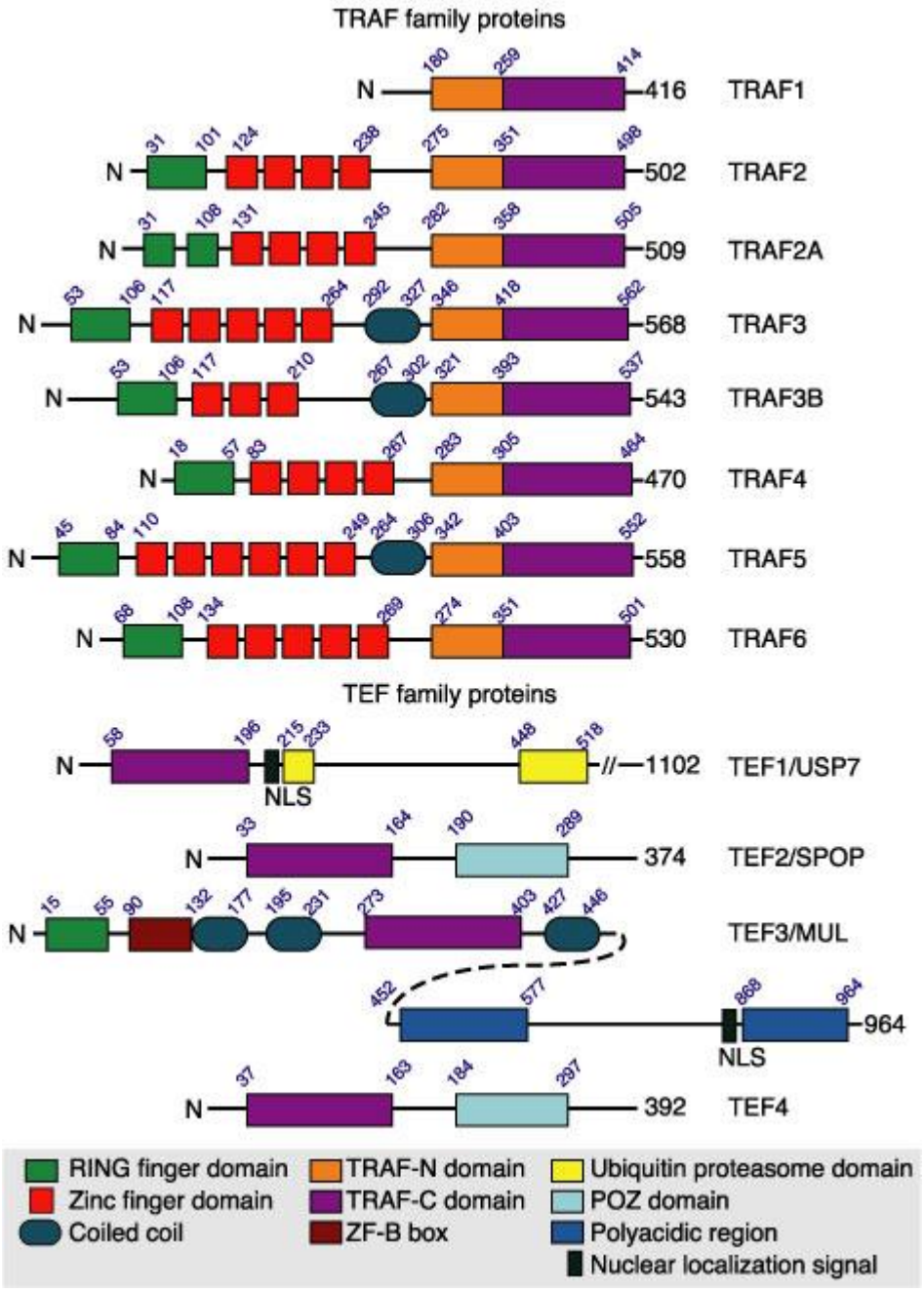


Figure 3: Schematic representation of the members of the TRAF family.

TAK1/NIK—IKK Kinases

Signaling to IKK downstream of RIPs and TRAFs depends on several kinases that have been implicated in NF-κB signaling pathways. In the case of canonical NF-κB pathways this role is largely fulfilled by TAK1 (TGFβ-activated kinase-1) (Sato et al., 2005; Shim et al., 2005). In noncanonical pathways NIK is instead required for IKKα activation and p100 phosphorylation (Senftleben et al., 2001; Xiao et al., 2001). Whether NIK and TAK1 function analogously to one another, that is as putative IKK kinases (IKK-Ks), is a matter of some debate. Despite the clear requirement for TAK1 in multiple signaling pathways to IKK, the mechanism of action of TAK1 in signaling to NF-κB remains unclear. First, genetic ablation of TAK1 reveals variable NF-κB activation deficiencies in different canonical signaling pathways. TNFR1 signaling to NF-κB appears completely abolished, while the effect on antigen receptor signaling is more controversial (Liu et al., 2006; Sato et al., 2005; Wan et al., 2006). Second, whether TAK1 directly serves as an IKK-K, or mediates activation through an intermediary kinase, MEKK3 for example, is not yet clear (Blonska et al., 2005; Li et al., 2006a). Third, even though the pathways triggered by LTβR lead to IKK through many of the same signaling intermediates, this pathway does not depend on TAK1 (Shim et al., 2005). Instead it seems that TAK1 generally functions in pathways that also require RIP for the activation of IKK. Indeed, RIP may be responsible for the recruitment of TAK1 (Blonska et al., 2005). TAK1 has been implicated in both antigen receptor and NOD signaling pathways, neither of which has yet been shown conclusively to depend on TRAF proteins, although there are indications that this is likely to be the case (Abbott et al., 2007; Hasegawa et al., 2007; Sun et al., 2004). Alternatively, it may be that some pathways that signal independently of RIPs, which to date includes mainly noncanonical pathways, may activate IKK without TAK1. Conversely, NIK can activate the noncanonical pathway in the absence of RIP proteins. NIK directly phosphorylates and activates IKKα and this model is supported by analyses of *NIK*−/− and *aly*/*aly* mice, which bear an inactivating point mutation in the NIK kinase (Hacker and Karin, 2006). Regulation of NIK is regulated by the combined action of TRAF proteins, as discussed above. In addition to TRAF3, cIAP1 and cIAP2 have been implicated as E3 ligases responsible for regulating constitutive NIK levels (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007). Degradation of cIAP downstream of noncanonical stimuli may function, like degradation of TRAF3, in leading to the accumulation of NIK and IKKα activation (Varfolomeev et al., 2007). However, the mechanism of cIAP regulation following receptor ligation needs to be further characterized.

In summary, a few common signaling components mediate activation of IKK under most circumstances. In the noncanonical pathway, TRAF and NIK are sufficient to activate IKKα in a NEMO-independent manner. However, the canonical pathway appears more complex. Generally canonical signaling relies on both TRAF and RIP proteins, as well as the kinase TAK1, although in certain pathways other proteins, e.g., IRAK1, may function analogously to RIP. It seems

that the key to this difference is likely to lie in binding of RIP, or analogous proteins, to NEMO, as both of these components may be universally required for canonical NF-κB activation. Whether this interaction allows TAK1 or another kinase to phosphorylate IKK or promotes IKK transautophosphorylation remains a matter of continuing debate. If there is no IKK-K in canonical pathways, however, then the exact function of TAK1 in IKK activation remains to be discovered.

Organization and Activation of the IKK Complex

Although exceptions have been reported, it is generally accepted that activation of NF-κB requires activation of either IKKα or IKKβ. It therefore follows that understanding the regulation of IKK activity is central to understanding the activation of NF-κB. Despite the significance of IKK, major gaps remain in our knowledge of the biochemistry of the IKK complex. However, several recent advances suggest that the field is on the verge of significant breakthrough into understanding the mechanism of IKK activation. The IκB kinase was first purified as a basally active, highmolecular-weight complex capable of phosphorylating serines 32 and 36 of IκBα (Chen et al., 1996). A stimulus-dependent kinase activity was subsequently identified by several groups and found to be composed of the two catalytic kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit NEMO (IKKγ) (DiDonato et al., 1997; Mercurio et al., 1997; Rothwarf et al., 1998; Woronicz et al., 1997; Yamaoka et al., 1998; Zandi et al., 1997; Regnier et al., 1997). IKKα and IKKβ, along with IKKi (IKKε) and TBK1, comprise the IKK family of proteins. IKKα and IKKβ share 52% overall sequence identity, with a greater degree of similarity in the catalytic domain (65%). NEMO is a 48 kDa protein that is not related to IKKα and IKKβ and contains a C-terminal Zn finger-like domain, a leucine zipper, and N-terminal and C-terminal coiled-coil domains (Figure 2). Targeted disruption of each of the IKK genes as well as transgenic and conditional knockout animals have been generated and extensively analyzed and reviewed recently (Gerondakis et al., 2006; Pasparakis et al., 2006). Initially the similarity between the IKKβ knockout and p65 knockout phenotypes argued for a central role for IKKβ in activation of p65 dimers via IκBα phosphorylation. Mice deficient in IKKα survive embryonic development but die perinatally due to multiple morphological defects, in particular aborted epidermal and skeletal development.

While initially it appeared that IKKα was dispensable for classical NF-κB activation, subsequent reports revealed the requirement for IKKα in multiple noncanonical NF-κB signaling pathways and perhaps some canonical signaling pathways as well (Solt et al., 2007; Takaesu et al., 2003). Furthermore, as discussed below, while not required for IκBα degradation in all pathways, IKKα likely plays an important role in NF-κB dependent gene expression in canonical signaling pathways. NEMO is required for signaling in all canonical NF-κB

pathways and NEMO-deficient mice also die embryonically of massive hepatocyte apoptosis. NEMO-deficient cells exhibit a more severe and broader loss of NF-κB activation than do IKKβ knockout cells, demonstrating that some canonical pathways are intact in the absence of IKKβ (Schmidt-Supprian et al., 2000; Solt et al., 2007). Therefore, it is more appropriate to categorize NF-κB pathways as canonical or noncanonical based on the requirement for NEMO or on the specific IκB protein that is phosphorylated and degraded/processed, e.g., IκBα, IκBβ, and IκBε for canonical and p100 for noncanonical, rather than on the requirement for IKKα or IKKβ.

B cell and NF-κB

BAFF-R-dependent activation of the alternative NF-κB pathway plays an essential role in mature B cell survival. Mutations leading to overexpression of NIK and deletion of the *TRAF3* gene are implicated in human multiple myeloma. NIK controls alternative NF-κB signaling by increasing the protein levels of its negative regulator TRAF3 in a dose-dependent fashion. This mechanism keeps NIK protein levels below detection, even when they cause B cell hyperplasia, so that contributions of NIK to B cell pathologies can easily be overlooked. B cells are central players in adaptive immune responses and immunity, mainly through their ability to differentiate into antibody-producing plasma cells and long-lived memory B cells upon antigen-encounter. Their survival and function critically depend on extracellular cues, which upon recognition by cell-surface receptors induce defined gene expression programs via activation of specific signal transduction cascades and transcription factors. Deregulation of these signaling pathways can lead to autoimmunity and lymphomagenesis. The NF-κB signal transduction cascade has emerged as a critical players in all of these process (Courtois G. et al. 2006; Hayden M.S. et al., 2006; Karin M. et al., 2002). In mature resting B cells, both pathways of NF-κB activation are induced and their generation and survival depends on both, demonstrated by gene disruption in the mouse at the level of transcription factors and signaling proteins (Sen R., 2006; Siebenlist U., 2005). It has been shown that the binding of B cell-activating factor of the TNF family (BAFF or BLyS) to BAFF-R (BR3), one of its three receptors, the others being transmembrane activator, calcium modulator, and cyclophilin ligand interactor (TACI), and B cell maturation antigen (BCMA) is required and sufficient to sustain the p100 processing needed for B cell survival (Siebenlist U., 2005). The main role of the alternative NF-κB branch in mature B cells appears to be the transmission of a survival signal elicited by BAFF, whose limited availability regulates the size of the peripheral B cell pool. Recent B cell-specific ablation studies of tumor necrosis factor receptor-associated factors (TRAFs) TRAF2 (Grech A.P. et al. 2004) and TRAF3 (Xie p. et al., 2007; Gardam S. et al., 2008) in mice have revealed striking phenotypic similarities to BAFF-transgenic mice, such as a pronounced B cell hyperplasia due to enhanced cell survival. On a

molecular level, lack of TRAF2 or TRAF3 prominently induces robust p100 processing, independent of the presence of BAFF (Grech A.P. et al. 2004; Xie p. et al., 2007; Gardam S. et al., 2008). The most straightforward explanation for this phenomenon is that the absence of TRAF2 or TRAF3 increases the protein concentration of NIK, as seen in TRAF3-deficient fibroblasts and transformed B cells (He J.Q. et al., 2007; He J.Q. et al., 2006; Liao G. et al., 2006), and suggested by the stabilization of NIK achieved through the removal of its TRAF3-binding domain (T3BD) (Liao G. et al., 2006). However, elevated amounts of NIK protein could not be detected in primary B cells lacking TRAF2 or TRAF3. The alternative pathway has been implicated in hematopoietic malignancies through chromosomal abnormalities leading to the production of truncated p100 proteins with diminished NF-κB-inhibitory ability. Recently, two independent studies uncovered genetic aberrations affecting components of NF-κB activation, mostly assigned to the alternative branch, in human multiple myeloma cell lines (HMCL) and in 9% (Annunziata C.M. et al., 2007) or 17% (Keats J.J. et al., 2007) of patient cohorts with multiple myeloma (MM). These aberrations led to the absence of negative regulators of NF-κB, such as TRAF3, TRAF2, cellular inhibitor of apoptosis proteins 1 and 2 (c-IAP1/2), and cylindromatosis protein (CYLD) or to overexpression of NIK. Collectively, these studies indicate that deregulation of the TRAF3-NIK axis might play an important role in lymphomagenesis.

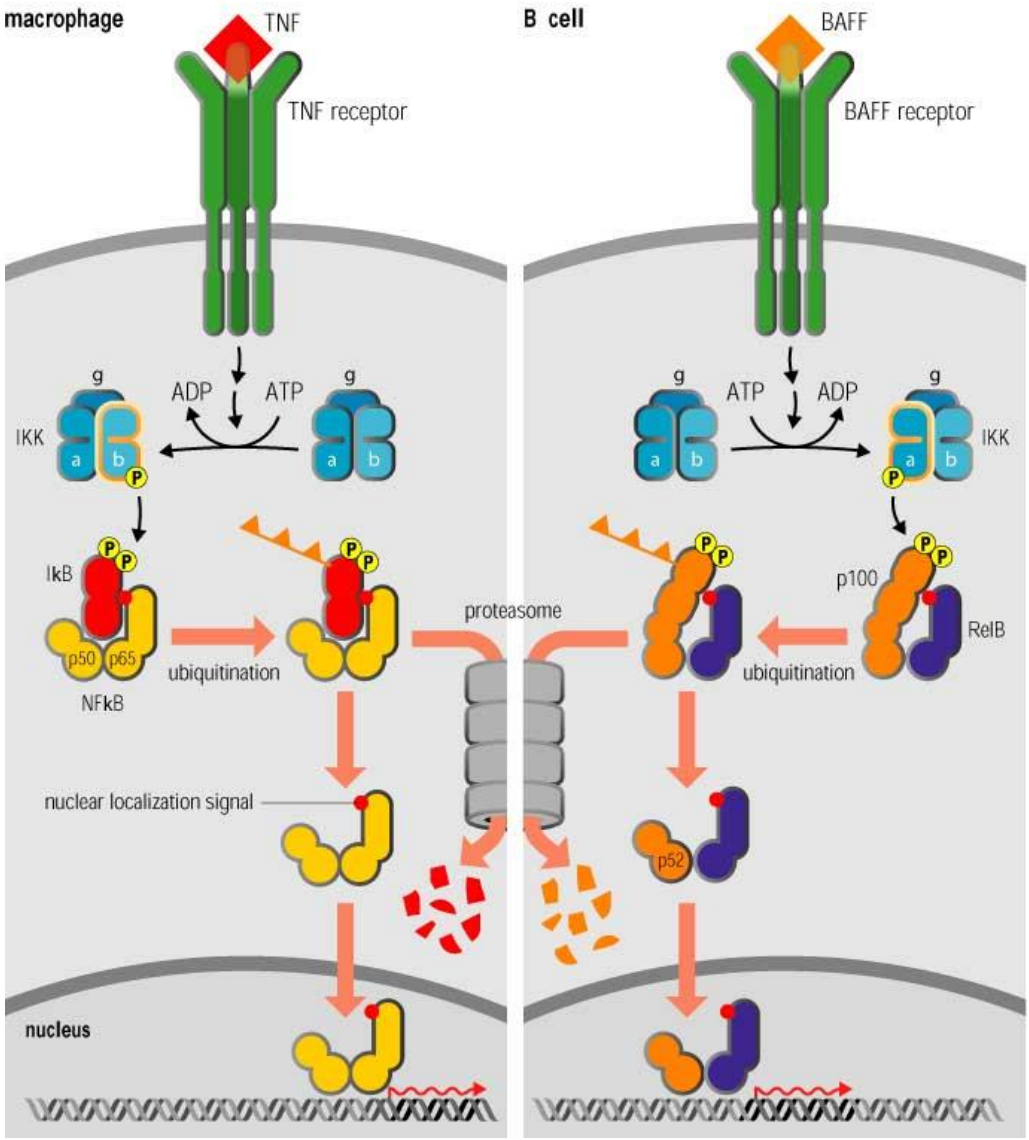


Fig.4: NF-kB signaling in Macrophage and B Cells

TRAF1

TRAF1 is unique among TRAFs because it lacks the “really interesting new gene” (RING) domain found near the NH2- terminal regions of TRAF2-6. Deletion of the RING domain, which contains a Zn2+-binding fold, from TRAF2, TRAF5, or TRAF6, revealed that the RING domain is critical for the activation of NF-κB or Jun NH2-terminal kinase (JNK) (Bradley J.R. et al., 2001). Moreover, RING-deleted (ΔRING) mutants of these TRAFs typically inhibit signal transduction by members of the TNFR family. Given the absence of a RING domain in TRAF1, it

may not be surprising that a recent paper from Tsitsikov and co-workers has provided in vivo evidence of a negative regulatory role for TRAF1 in mice by demonstrating enhanced TNF signaling in TRAF1-deficient mice (Tsitsikov E.N. et al., 2001). However, when compared with other observations concerning TRAF1, one wonders whether there might be more to the story of this unique TRAF family member.

The expression of TRAF1 is the most restricted among TRAFs; TRAF1 is found almost exclusively in activated lymphocytes, dendritic cells, and certain epithelia (Zapata J.M. et al., 2000). TRAF1 has been reported to associate directly or indirectly with multiple TNFR family members. Though results are sometimes based on overexpression experiments, TRAF1 reportedly also binds several intracellular proteins, including adaptor proteins such as TNFR-associated death domain protein (TRADD), TRAF-associated and NF-κB activator (TANK, also known as I-TRAF), and TRAF-interacting protein (TRIP); protein kinases such as NF-κB-inducing kinase (NIK), receptor-interacting protein (RIP) and RIP2 (Cardiak); the NF-κB inhibitory protein A20; and the apoptosis-suppressors inhibitor of apoptosis 1 (cIAP1), cIAP2, and FADD-like interleukin-1β converting enzyme (FLICE)-like inhibitory protein (FLIP). Additionally, TRAF1 forms heteromers with TRAF2. TRAF1 can also be recruited to the TNFR1 and TNFR2 through its interaction with TRADD and TRAF2, respectively, where it is also found associated with cIAP1 and cIAP2.

This plethora of interacting partners positions TRAF1 for multiple possible functions and suggests that absence of this protein should produce several defects in cytokine signal transduction networks. Surprisingly, however, Tsitsikov and coworkers find that TRAF1 knockout mice are phenotypically normal in appearance. TRAF1-/- mice have no apparent defects in development of either T cells or B cells, and have normal numbers of lymphocytes in peripheral lymphoid organs, unimpaired antibody responses to both T-dependent and T-independent antigens, and typical kinetics of lymphocyte deletion and expansion in response to superantigen. Only the inguinal lymph nodes of TRAF1-deficient mice showed significant differences compared to wild-type littermates, having a larger number of lymphocytes and an increased T/B cell ratio. These results stand in sharp contrast to the those of gene knockouts for TRAF2, TRAF3, TRAF4, and TRAF6; striking developmental defects in these mice are evident.

AIM OF THE STUDY

BAFF-Receptor dependent activation of the alternative NF-κB pathway plays an essential role in mature B cells survival. Mutations leading to overexpression of NIK and deletion of the TRAF3 gene are implicated in human lymphoma and myeloma. This overexpression of NIK leads to an uncontrolled activation of NF-κB2 that seems to be the cause of tumoral transformation. In the same cells the expression of TRAF1 is upregulated. For this reasons the aims of our study is to correlate this two events and to clarify some mechanisms of function of NF-κB2.

MATERIALS AND METHODS

1 - Cell Culture and Biological Reagents

HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 1% glutamine. Lymphocytes B cells U266 (myeloma) were maintained in RPMI medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 1% glutamine. Antibodies used for this study were: FLAG epitope (Sigma), HA epitope, TRAF1 polyclonal, GST, p52 polyclonal, Actin and Tubulin (Santa Cruz Biotechnologies), Anti-NIK polyclonal antibodies were generated in rabbits, by using a recombinant peptide. Human BAFF (Peprotech Inc.) was used at 100 ng/ml.
Human TRAF1, Baff receptor, NIK, TRAF3 and p100/p52 was amplified by PCR from human PBL cDNA library (BD clontech) and cloned into pcDNA3-FLAG, –HA and pGEX2T vectors (Invitrogen) for expression in mammalian cells. All deletion mutant were prepared by conventional PCR and cloned into pcDNA3-FLAG, –HA.

2 - In Vitro Translation and GST Pull-down Assays

In vitro transcription and translation were carried out with 1 μg of FLAG-TRAF1 and FLAG-TRAF3 constructs according to the TNT Quick Coupled Transcription/Translation System protocol (Promega). GST-BAFF receptor fusion protein was produced and purified as described (33). GST pull-down assays were performed by incubating an aliquot of GST-BAFF receptor bound to glutathione-Sepharose beads (Amersham Biosciences) together with 10, 20 e 30 μl of *in vitro* translated FLAG-TRAF1 and 10 μl of FLAG-TRAF3 protein in phosphate-buffered saline, 1% Triton X-100 buffer (including Complete Protease Inhibitor mixture (Roche)) for 2 h at 4 °C. Beads were then washed five times with the same buffer, resuspended in Laemmli buffer, and run on a SDS-polyacrylamide gel before autoradiography.

3 - Transfection, Immunoprecipitation, and Luciferase Assay

LipofectAMINE-mediated transfections were performed according to the manufacturer's instructions (Invitrogen). All transfections included supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each dish culture.
For immunoprecipitation of transfected proteins, HEK293 cells (3 x 10^6) were transiently transfected and 24 h after transfection cells were lysed in Triton X-100

lysis buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and Complete Protease Inhibitor mixture). After an additional 15 min on ice, cell extracts were centrifuged for 10 min at 14,000x*g* at 4 °C and supernatants were incubated for 3 h at 4 °C with anti-FLAG antibodies bound to agarose beads (M2, Sigma). The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.
For luciferase assay, HEK293 cells ($3x10^5$) were seeded in 6-well plates. After 12 h cells were transfected with 0.5 μg of Ig-κB-luciferase reporter plasmid and various combinations of expression plasmids. 24 h after transfection cells extracts were prepared and reporter gene activity was determined via the luciferase assay system (Promega). Expression of the pRSV-β-galactosidase vector (0.25 μg) was used to normalize transfection efficiencies.

4 - In Vivo Ubiquitination and De-ubiquitination Assays

HEK293 cells (3×10^6) were co-transfected with expression vectors containing HA-epitope-tagged ubiquitin (1 μg) and various combinations of expression plasmids. 24 h after transfection, cell lysates were prepared as above and analyzed for polyubiquitination of TRAF3 either by Western blot anti-TRAF3 (FLAG) on total extracts or by immunoprecipitating FLAG-TRAF3 with anti-FLAG beads followed by Western blot anti-HA-ubiquitin.

5 - TRAF1 Short hairpin RNA (shRNA) Expression Vectors

To knockdown TRAF1 expression in Myeloma B cells L1236, we have used "SHVRS MISSION shRNA Lentiviral Particles" of Sigma. shRNA Lentiviral Particles are transduction -ready viral particles for gene silencing in mammalian cells (human or mouse) including both dividing, non-dividing, and primary cell types. After transduction, stable cell lines expressing the shRNA was selected with puromycin.
MISSION™ TRC shRNA Target Set
TRCN0000056883:CCGGGCAGTCTCAATGGGTCAGAAACTCGAGTTTCTGACCCATTGAGACTGCTTTTTG
TRCN0000056884:CCGGGCCTTCTACACTGCCAAGTATCTCGAGATACTTGGCAGTGTAGAAGGCTTTTTG
TRCN0000056885:CCGGCGTGTGTTTGAGAACATTGTTCTCGAGAACAATGTTCTCAAACACACGTTTTTG
TRCN0000056886:CCGGGATGAGAATGAGTTTCCCTTTCTCGAGAAAGGGAAACTCATTCTCATCTTTTTG
TRCN0000056887:CCGGCATTGTGGAGACCAGCACTTACTCGAGTAAGTGCTGGTCTCCACAATGTTTTTG

6 - *RNA extraction and RT–PCR assays*

The U266 cells lines were stimulated with Baff Ligand (Peprotech) for the hours indicated. Total RNA (1 µg) isolated from U266 cells lines by the Chomczynski and Sacchi method (Chomczynski and Sacchi 1987). After checking the integrity of RNA by electrophoresis on agarose gel, an aliquot of total RNA was incubated for 15 min at 37°C with 20 U of RNAse-free DNAse I (Promega, Madison, WI) to digest contaminating genomic DNA.
RT-PCR experiments were performed in accordance to standard methods (Innis at *al*. 1990). 0,5 µg of cDNA were amplified in 50 µl of PCR buffer (Promega) containing 15 pmol of TRAF1 specific forward and reverse primers (see below). After a first step of denaturation for 2 min at 95°C, 25 or 35 cycles of cDNA amplification from cells lines extracts were performed as follows: 30 sec at 95°C, 30 sec at 55°C and 30 sec at 72°C with a final extension of 5 min at 72°C. The last 20 cycles of amplification were carried out adding 15 pmol of human GAPDH forward and reverse specific primers to the PCR mixture. The following TRAF1 primers were used: forward 5'-cta ccg ggc acc ctg ctc cg-3' and reverse 5'-gca ttg tgg aga cca gca ctt ag-3'. The cDNA concentrations were normalized by GAPDH mRNA content.

RESULTS AND DISCUSSION

Identification of TRAF1 as a specific BAFF-R-interacting protein

The intracellular signaling pathway mediated by BAFF-R is only partially known. Similar to other members of the TNFR family, BAFF-R does not have intrinsic enzymatic activity, and is believed to transduce signals through physical interaction with downstream signaling proteins. Because TRAF proteins are involved in signaling by many members of the TNFR family, we decided to investigate a potential role for TRAF1 in BAFF-R signaling. To determine whether TRAF1 is associated with cytosolic domain of BAFF-R in mammalian cells, expression plasmids encoding the intarcellular domain of BAFF Receptor, and Flag-tagged TRAF1 were transfected into 293 cells. Coimmunoprecipitation experiments indicated that BAFF-R interacted with TRAF1 (Fig. 5A). In order to confirm the interaction and to demonstrate a direct interaction between BAFF Receptor and TRAF1, we performed GST-pull-down assay, recombinant protein GST-BAFF-R was purified in *E. Coli* (DH5α) and Flag-tagged TRAF1 and TRAF3 were produced with Reticolcytes Trascription/Traslation System. The interaction between BAFF Receptor and TRAF3 was used as positive control of interaction. As shown in figure 5B TRAF1 was directly binding cytoplasmic domain of BAFF Receptor. This experiment also suggests that binding of TRAF1 and TRAF3 to the receptor involved different domains of the receptor. In fact, increasing amount of TRAF1 was not affecting the binding of TRAF3 to the receptor.

Fig. 5A

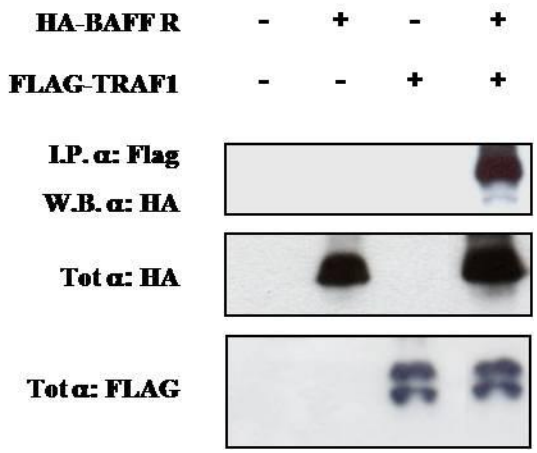


Fig. 5B

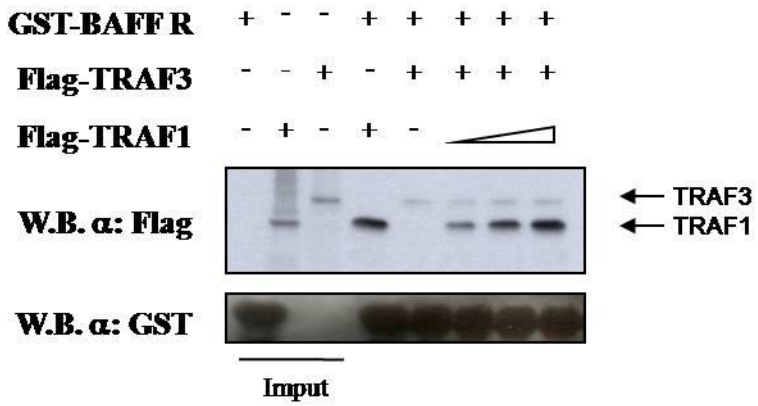


Figure 5: BAFF-R interacts with TRAF1 in 293 cells. A, 293 cells were transfected with expression plasmids for C-terminal HA-tagged BAFF-R and the indicated Flag-tagged TRAF1 protein. Cell lysates were immunoprecipitated with anti-Flag Ab. The immunoprecipitates were analyzed by Western blot with anti-HA (*upper panel*). Expression of BAFF-R and TRAF1 proteins was confirmed by Western blots with anti-HA (*middle panel*) and anti-Flag (*lower panel*) Abs, respectively. B, Confirmation of interaction with pull-down. Purified GST-BAFF Receptor was incubated with FLAG-TRAF1 or TRAF3 and immunoprecipitated with Glutation Sepharose 4B and analyzed by W.B. with anti-Flag (*upper panel)* and with anti-GST (*lower panel*).

Domain mapping of the interaction between BAFF Receptor and TRAF1

TRAF proteins interact with members of the TNFR family through their TRAF domains. To determine the regions of TRAF1 that are required for interaction with BAFF-R, we generated a series of C-terminal HA-tagged deletion mutants of the TRAF1 (Fig. 6A). Transient transfection of 293 cells and coimmunoprecipitation experiments suggest that the amino acid between 300-350 are required for interaction with BAFF-R (Fig. 6B).

Fig. 6A

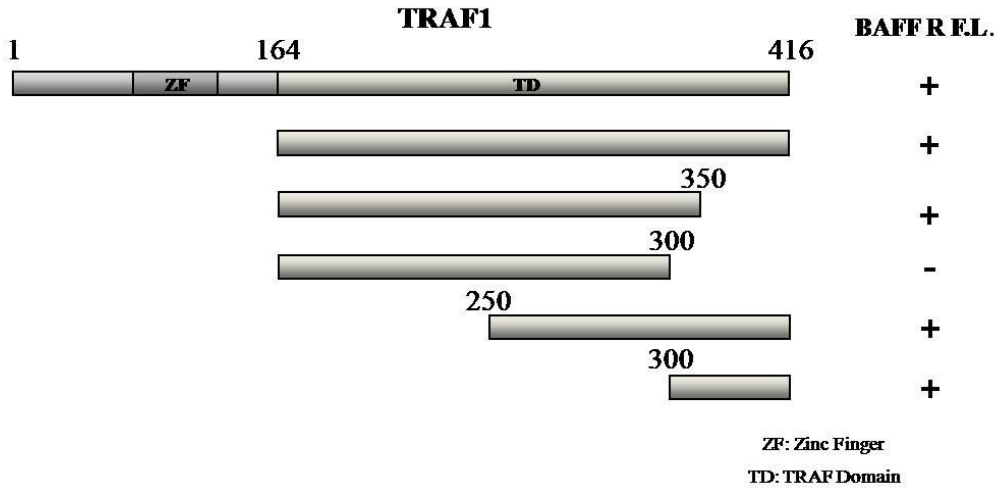


Fig. 6B

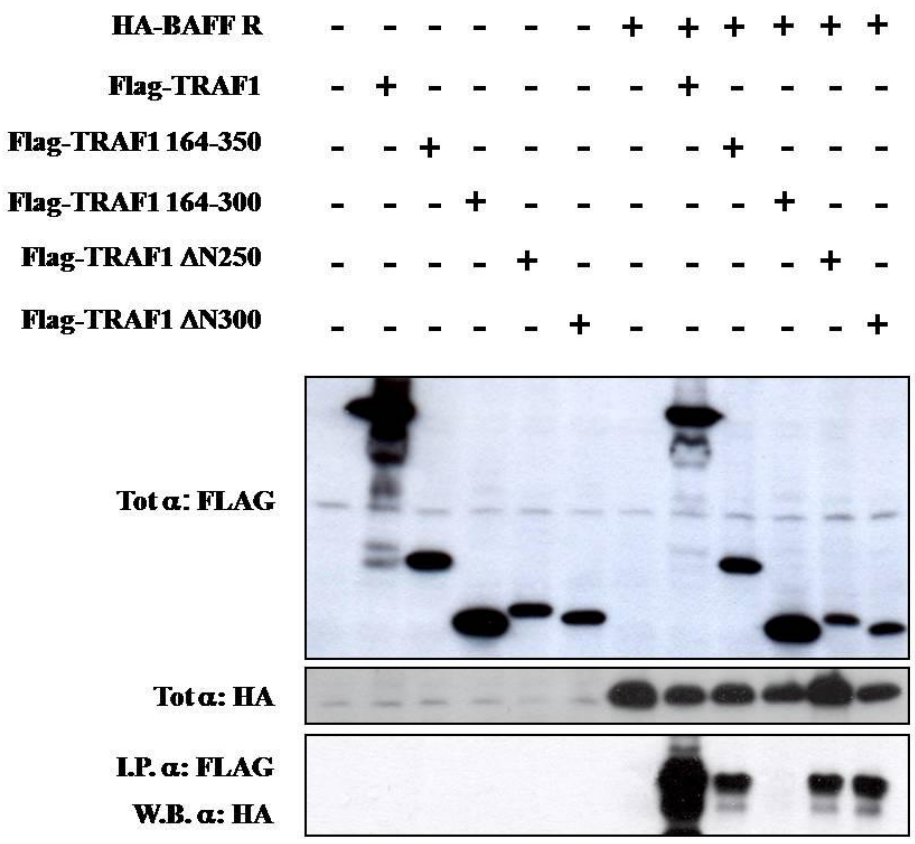
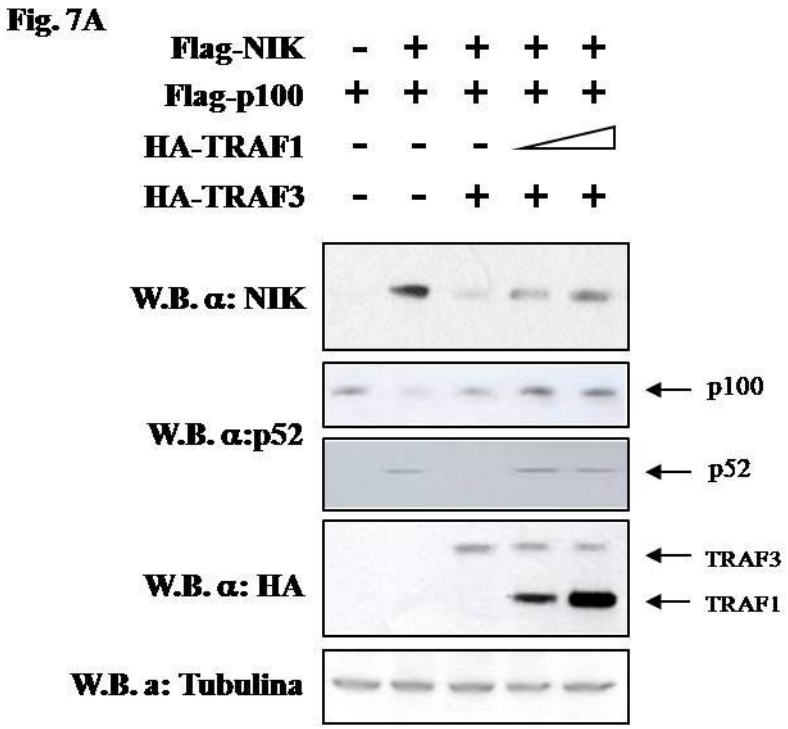


Figure 6. Mapping of TRAF1 interaction with BAFF-R. *A*, A schematic presentation of the TRAF1 deletion mutants and their interaction with BAFF-R. TD, TRAF Domain; ZF, Zinc Finger. B, Coimmunoprecipitation between BAFF-R and TRAF1 mutants. 293 cells were transfected with

expression plasmids for HA-tagged BAFF-R and the indicated C-terminal Flag-tagged TRAF1 mutants. Cell lysates were immunoprecipitated with anti-Flag Ab and the immunoprecipitates were analyzed by Western blot with anti-HA Ab (*lower panel*). Expression of TRAF1 mutants and BAFF-R was confirmed by Western blots with anti-Flag (*upper panel*) and anti-HA (*middle panel*) Abs, respectively.

TRAF1 positively regulates NF-κB activation by inhibiting TRAF3 protein expression

TRAF3 is a negative regulator of p100 processing. To determine whether TRAF1 is involved in degradation of NIK by TRAF3, we transfected, in 293 cells, NIK, HA-tagged TRAF3 and increasing amount of HA-TRAF1. The cell lysates were analyzed by Western blot with anti-HA and anti-NIK Abs. As shown in figure 7A, ectopic expression of TRAF3 decreased the level of NIK and blocks processing of p100. However, in the presence of increasing amount of TRAF1, TRAF3 is decreased, the level of NIK is stabilized and processing of p100 is restored. The positive effect played by TRAF1 on p100 processing was farther confirmed by evaluating the effect of TRAF1 in the activity of an alternative pathway specific κB reporter (Bonizzi at al. 2004 EMBO J.). As shown in figure 7B, the NIK dependent alternative pathway activation, was blocked by TRAF3 and restored in the presence of TRAF1.
These results strongly suggest that TRAF1 is a positive regulator of the alternative pathway, interfering with the TRAF3-mediated NIK degradation.



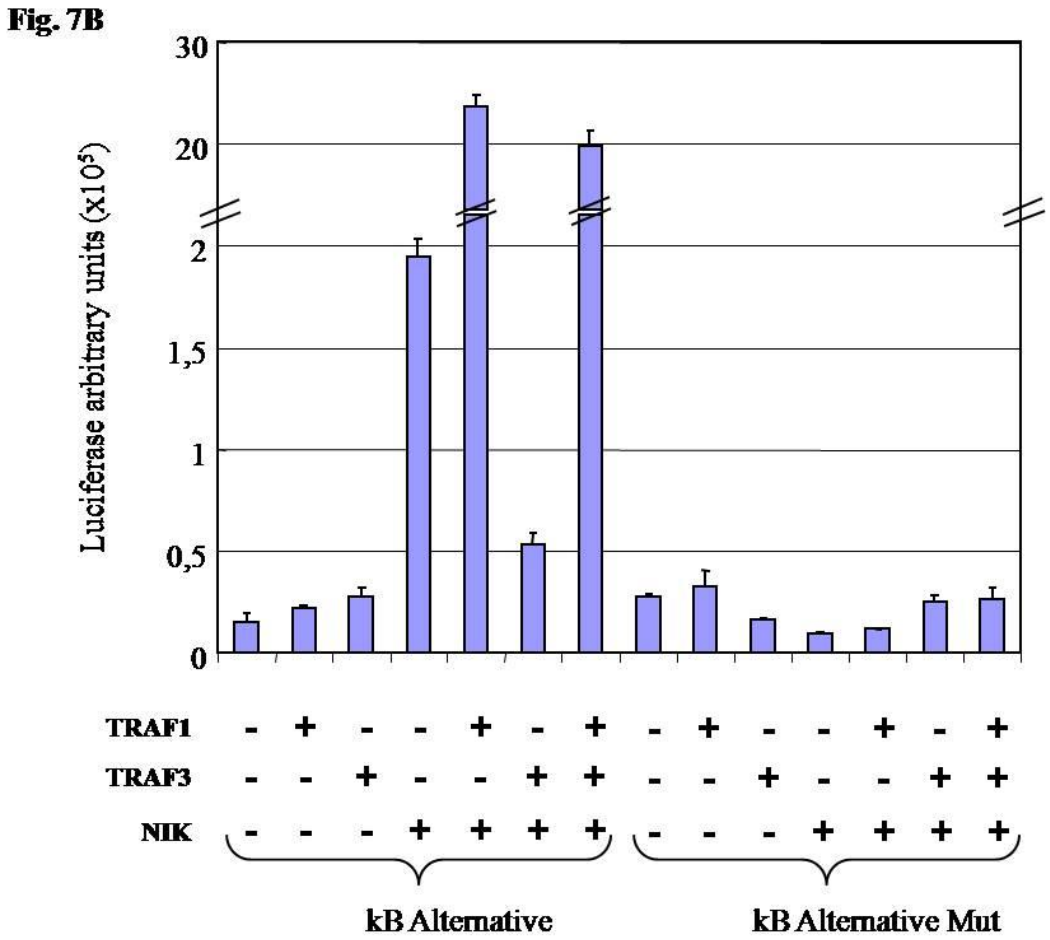


Figure 7. TRAF1 induce decrease of TRAF3 level and consequently a stabilization of NIK and an increase of p100 processing. *A*, 293 cells were transfected with expression plasmids for NIK, HA-TRAF1, HA-TRAF3 and FLAG-p100. Cell lysates were analyzed by Western blot with anti-NIK (*upper panel*), anti-HA and anti-FLAG (*middle panel*) Abs. The Western blot anti-Tubulin was used as a control for total proteins expression. B. TRAF1 induce activation of alternative pathway of NF-κB. Relative reporter activity was evaluated in HEK-293T cells co-transfected with the Ig-κB-luciferase alternative and mutated plasmid and the indicated expression vectors. Values shown in arbitrary units represent the mean ± S.D. of three experiments done in triplicate, normalized for β-galactosidase expression of a co-transfected pRSV-β-galactosidase plasmid.

Induction of TRAF1 upon BAFF ligand stimulation in myeloma B cells

In order to understand if expression of TRAF1 was inducible after BAFF stimulation U266 cells were stimulated with BAFF, and the expression of TRAF1 was evaluated by semi-quantitative PCR. As shown in figure 8A BAFF treatment caused a marked increase in TRAF1 mRNA expression. The increased expression of TRAF1 correlated with p100 processing (Fig. 8B).

Fig. 8A

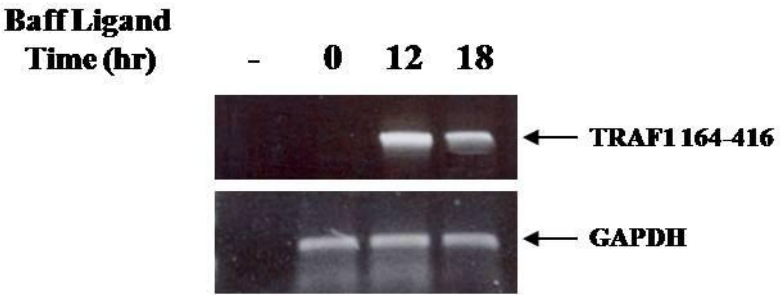


Fig. 8B

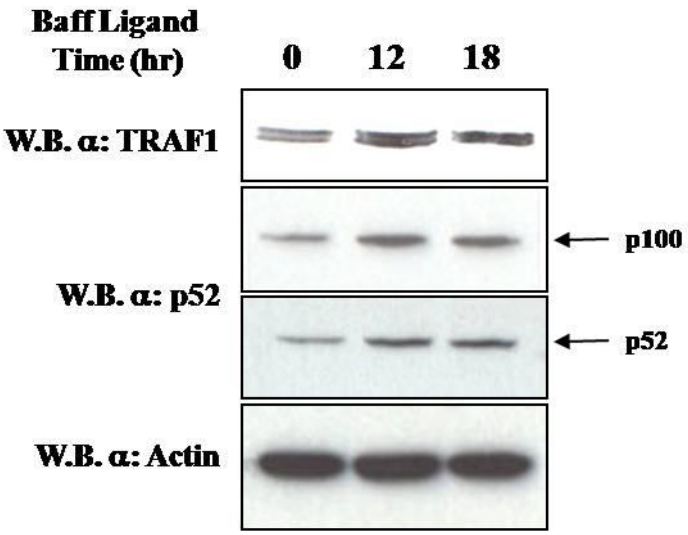


Figure 8: Induction of TRAF1 upon BAFF ligand stimulation in myeloma B cells. A. U266 cells were stimulated with 100 ng/ml of BAFF ligand for the indicated hours. Then it was extracted mRNA, converted in cDNA by RT-PCR and run on 1% agarose elettrophoresis gel. The GAPDH (housekeeping gene) cDNA was used for a control of total amount of cDNA present in the various preparations. B. An aliquot of the same cells used for the extraction of mRNA was lysated and analyzed by W.B. anti-TRAF1 (upper panel) and anti-p52 (middle panels) Abs. The Western blot anti-Actin was used as a control for total proteins expression.

TRAF1 down-regulation decreases p100 processing and affects B cell survival

To further demonstrate the positive role played by TRAF1 in regulating p100 processing, we decreased its expression in the lymphoma B cell line L1236 by shRNA. We utilized 3 different shRNA constructs named Cl1, Cl3 and Cl5 and 1 scramble shRNA as control. Cells were transduced with two different amount of lentiviral particles carrying the shRNA. As shown in figure 9, all the shRNA used decreased the expression of TRAF1, albeit at different level. As expected the decreased in TRAF1 expression caused a decrease in p100 processing.

Fig. 9

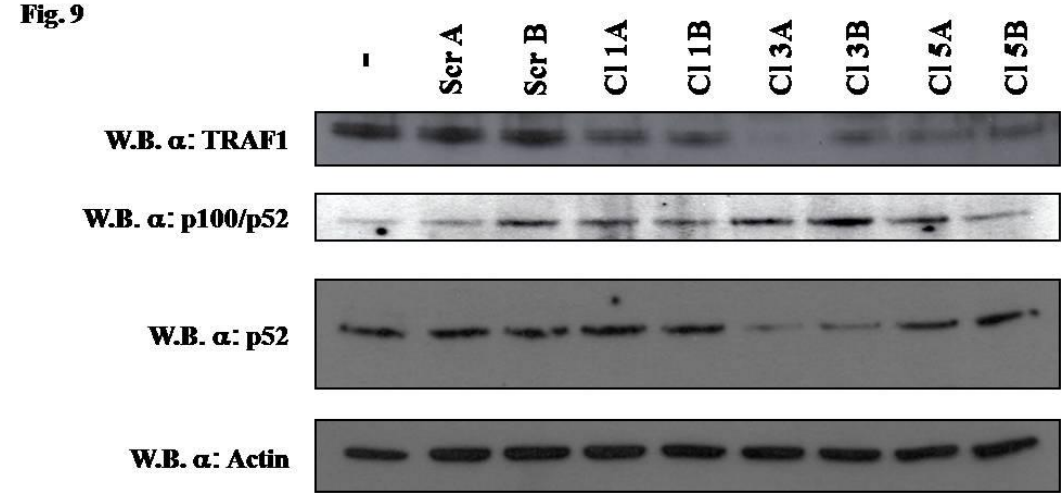


Figure 9: The interference of TRAF1 induces the decrease of p100 processing. L1236 cells were infected with Lentiviruses leads shTRAF1 interference constructs Cl1, Cl3, Cl5 and Sc. (scramble)in two different amounts A and B. Then cells were treated with 5 µg/ml of puromycin for kill untransduced cells. The survived cells were expanded and then subjected to a W.B. anti TRAF1(upper panel), p100 and p52 (middle panel). The Western blot anti-Actin was used as a control for total proteins expression.

The constitutive activation of the alternative pathway is important for B cell survival. Then we investigated the survival of the B lymphoma cell line L1236 transduced with shRNA for TRAF1. As shown in figure 10 the survival of the B cell line L1236 is decreased in absence of TRAF1.
Taken together these results demonsytrated that TRAF1 is a positive regulator of p100 processing, and plays a role in controlling survival of lymphoma cells.

Fig. 10

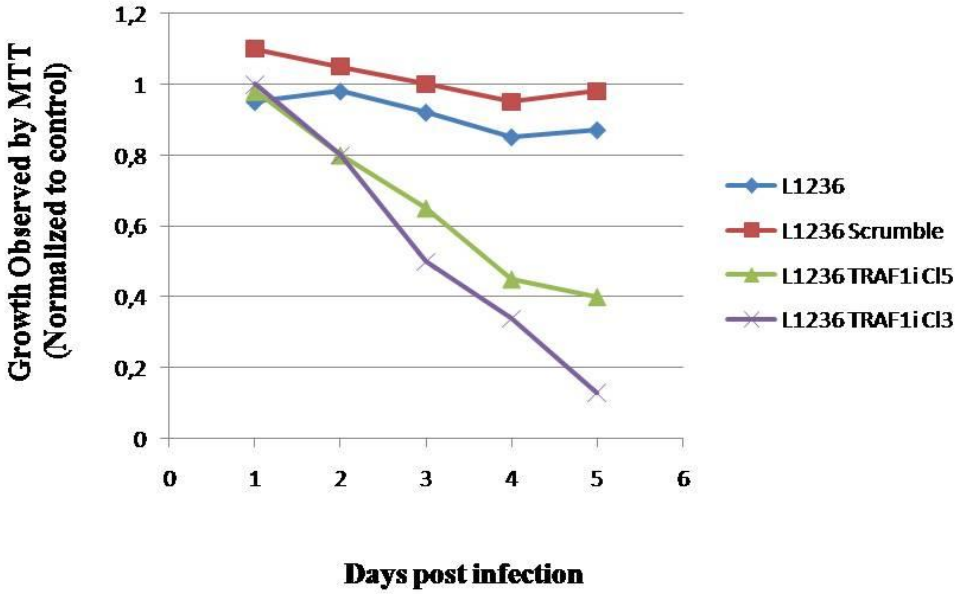


Figure 10: TRAF1 down-regulation affects B cell survival. The L1236 were transduced with a lentivirus expressing the TRAF1 shRNA. Live cells were enumerated by MTT for the indicated number of days.

DISCUSSION

In the present paper we identify TRAF1 as a Baff-receptor interacting protein, and present evidence for a role of TRAF1 as a positive regulator of the NF-κB alternative pathway. Activation of the NF-κB alternative pathway is essential for the organization of the secondary lymphoid tissue, and deregulation of this pathway has been detected in multiple myeloma cell lines and patients. These mutations affect both negative regulator (TRAF2, TRAF3, c-IAP1/2), and positive regulator (NIK) of the pathway.

In the current model of activation of the alternative pathway, TRAF3 constitutively binds to and ubiquitinates the protein kinase NIK. After receptor engagement, TRAF3 is degraded, the level of NIK increases, and then NIK may phosphorylate IKKα to activate the complex. However, how engagement of the receptors that control B cell survival and development (such as CD40 and BAFF-R) regulates this process is currently unknown. Protein synthesis is required to activate the pathway, indeed p100 processing take place few hours after receptor engagement and is blocked by protein synthesis inhibitor. In our experimental model, TRAF1 is induced by BAFF-R triggering and its induction correlates with the increased p100 processing. In addition, we present evidence that the simple overexpression of TRAF1 decreases the level of TRAF3 and stabilizes NIK, thus mimicking receptor triggering. How TRAF1 interfere with TRAF3 function? A possible explanation might be that TRAF1 compete with TRAF3 for binding to the receptor. However, our data suggest that this is not the case, as increasing amount of TRAF1 does not displace TRAF3 from binding to the receptor (Fig 12 and data not shown). Another possibility is that TRAF1 displaces TRAF3 from NIK, thus preventing its ubiquitination and degradation. We are currently exploiting this possibility also if, at least in transfected cells, binding of TRAF3 to NIK is not affected by TRAF1 overexpression. There is another intriguing possibility: in the presence of TRAF1, TRAF3 became substrate for another ubiquitin ligase activity, leading to TRAF3 degradation, and NIK stabilization. Indeed, very recently, it has been recently proposed by Karin and colleagues, and by Chen and colleagues that TRAF3 degradation is mediated by a multimeric complex containg c-IAP1 and c-IAP2. This result suggest that TRAF1 may function to bridge interaction between IAPs and TRAF3. We are currently investigating if TRAF1 is part of this complex.

The present work also demonstrate that blocking expression of TRAF1 by shRNA in myeloma cells, blocks processing of p100 thereby affecting survival of myeloma cells. Elegant work recently found that a subgroup of multiple myeloma cells expresses elevated alternative pathway NF-κB activity, owing to amplification of NIK or mutation in TRAF3. This result confirm the central role played by the alternative NF-kB pathway in lymphocyte survival, and suggest that amplification of TRAF1 may also be responsible for a subgroup of multiple myeloma. Indeed, TRAF1 mRNA and protein levels are typically increased in B cell malignancies, including non-Hodgkin lymphomas, chronic lymphocytic

leukemias, as well as the Reed-Stemberg cells of Hodgkin disease. On the other hand, although T cells from TRAF1-null mice exhibited enhanced proliferation in vitro, their B cells have normal proliferation rates upon IgM or CD40 binding. In addition, TRAF1 transgenic mice that overexpress this protein in T cells, have normal basal and CD3-stimulated proliferation compared to control littermates, suggesting that overproduction of TRAF1 does not alter CD3-mediated T cell proliferation. However, in the same experimental setting, the TCR mediated-apoptosis is reduced. How do we reconcile these disparate observation? It is very possible that the difference in cell context may provide an explanation. However, it is possible that in vivo the simple overexpression of TRAF1 is not sufficient to sustain proliferation and/or resistance to apoptosis in normal B cells. Perhaps, mating of TRAF1 transgenic mice that overexpress this protein in B cells, with oncogene-expressing transgenic mice that develop B cell malignancies will shed some light on these questions.

Collectively, our results in addition to identify TRAF1 as a positive regulator of the alternative NF-kB activation pathway, identify TRAF1 as a potential target for treatment of myeloma.

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APPENDIX: ORIGINAL PAPERS

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ABIN-1 Binds to NEMO/IKKγ and Co-operates with A20 in Inhibiting NF-κB*[S]

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Nuclear factor κB (NF-κB) plays a pivotal role in inflammation, immunity, stress responses, and protection from apoptosis. Canonical activation of NF-κB is dependent on the phosphorylation of the inhibitory subunit IκBα that is mediated by a multimeric, high molecular weight complex, called IκB kinase (IKK) complex. This is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO/IKKγ. The latter protein is essential for the activation of IKKs and NF-κB, but its mechanism of action is not well understood. Here we identified ABIN-1 (A20 binding inhibitor of NF-κB) as a NEMO/IKKγ-interacting protein. ABIN-1 has been previously identified as an A20-binding protein and it has been proposed to mediate the NF-κB inhibiting effects of A20. We find that both ABIN-1 and A20 inhibit NF-κB at the level of the IKK complex and that A20 inhibits activation of NF-κB by de-ubiquitination of NEMO/IKKγ. Importantly, small interfering RNA targeting ABIN-1 abrogates A20-dependent de-ubiquitination of NEMO/IKKγ and RNA interference of A20 impairs the ability of ABIN-1 to inhibit NF-κB activation. Altogether our data indicate that ABIN-1 physically links A20 to NEMO/IKKγ and facilitates A20-mediated de-ubiquitination of NEMO/IKKγ, thus resulting in inhibition of NF-κB.

NF-κB is a ubiquitously expressed family of transcription factors that controls the expression of numerous genes involved in immune and inflammatory responses (1). NF-κB also plays an important role during cellular stress responses, due to its anti-apoptotic and proliferation-promoting functions (2). Aberrant activation of NF-κB is a major hallmark of several inflammatory diseases such as arthritis (3, 4), and a variety of human cancers (5, 6). In resting cells, NF-κB is sequestered in the cytoplasm in an inactive form by members of the inhibitory family of IκB proteins (1). Various stimuli including pathogens, pathogen-related factors, and cytokines lead to phosphorylation of the inhibitory subunit IκBα on specific serine residues (Ser^{32} and Ser^{36}) (7) catalyzed by two IκB kinases (IKKs),[3] namely IKKα and IKKβ (8–12). This step marks the IκB protein for ubiquitination and subsequent degradation through a proteasome-dependent pathway (1). The active NF-κB is then free for translocation to the nucleus, where it binds the κB sequences present in the promoters of responsive genes.

IKKα and IKKβ reside in a larger kinase complex (700–900 kDa), called the IκB kinase complex (IKK complex), that also contains the essential regulatory subunit NEMO (also known as IKKγ) (13, 14). Genetic studies suggest that NEMO/IKKγ is absolutely required for the activation of IKKs and NF-κB in response to different stimuli (13, 15). NEMO/IKKγ contains several coiled-coil domains, a leucine zipper, and a C-terminal zinc finger domain. These motifs are required for the correct assembly of the IKK complex (13) and recruitment of upstream signaling mediators (16). Numerous proteins have been demonstrated to interact with NEMO/IKKγ, as the kinase RIP and the inhibitor of NF-κB A20 (17), the viral trans-activator TAX (18–20), and the adaptor proteins CIKS/Act-1, TANK, and CARMA (21–23). Therefore, NEMO/IKKγ represents the point where most NF-κB signaling pathways converge. Despite this information, the molecular mechanism regulating IKK complex function is not fully understood.

Ubiquitin conjugation has been most prominently associated with protein degradation through a proteasome-dependent pathway, but it is becoming increasingly evident that ubiquitination plays a key role in the signal transduction pathway leading to activation of NF-κB (24, 25). Recent reports show that lysine 63-linked ubiquitination of NEMO/IKKγ is an important step for the activation of IKKs and NF-κB following various stimuli, such as TNF, lipopolysaccharide, and antigen receptor (26–28). In contrast, the tumor suppressor CYLD is reported as a negative regulator of NF-κB by specific de-ubiquitination of NF-κB signaling molecules, such as TRAF2, TRAF6, and NEMO/IKKγ (29). Also A20 functions as an inhibitor of the NF-κB pathway by removing Lys^{63}-linked ubiquitin chains from RIP, an essential mediator of the proximal TNF-Receptor-1 signaling complex. Then A20 targets RIP for Lys^{48}-linked polyubiquitination and proteasomal degradation (30). Furthermore, A20 terminates Toll-like receptor-induced NF-κB signaling, by cleaving ubiquitin chains from TRAF6 (27). The central role played by A20 in terminating NF-κB activation is further demonstrated by the fact that $A20^{-/-}$ mice develop severe inflammation and cachexia, are hypersensitive to both lipopolysaccharide and TNF, and die prematurely (27, 31). Here we used NEMO/IKKγ as bait in yeast two-hybrid screening, and identified ABIN-1 (A20 binding inhibitor of NF-κB) as a NEMO/IKKγ-interacting protein.

EXPERIMENTAL PROCEDURES

Cell Culture and Biological Reagents—HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 1% glutamine.

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This work is dedicated to the memory of Prof. Serafino Zappacosta.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[3] The abbreviations used are: IKK, IκB kinases; TNF, tumor necrosis factor; ABIN-1, A20 binding inhibitor of NF-κB; HA, hemagglutinin; GST, glutathione *S*-transferase; siRNA, small interfering RNA; CIKS, connection to IκB kinase and SAPK.

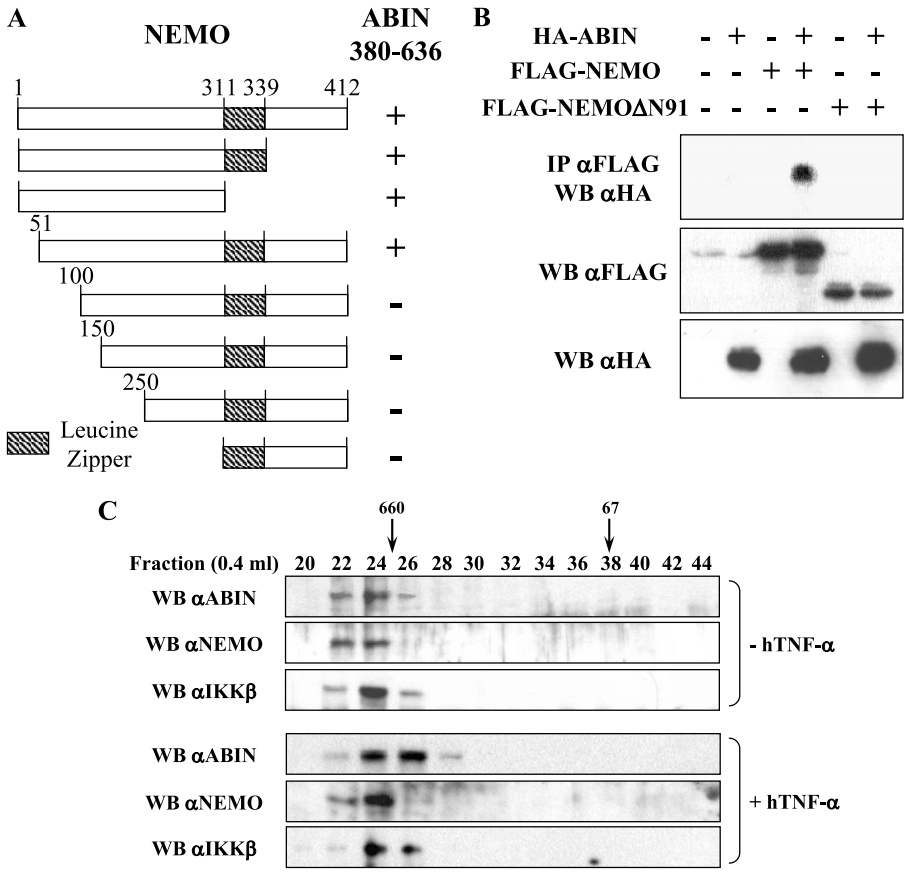

FIGURE 1. **Mapping of the ABIN-1 interaction domain on NEMO/IKKγ.** *A*, mapping of the NEMO/IKKγ-ABIN interaction by yeast two-hybrid experiments. The NEMO/IKKγ constructs are schematically represented. The interaction of the NEMO/IKKγ constructs with the clone isolated by yeast two-hybrid screening (ABIN 380–636) is indicated by the *plus sign*. *B*, *in vivo* mapping of the NEMO/IKKγ-ABIN interaction. HEK293 cells were transfected with the indicated combinations of expression constructs encoding HA-ABIN and either FLAG-NEMO/IKKγ or FLAG-NEMOΔN91. Cell extracts were analyzed by immunoblotting either directly or after immunoprecipitation with anti-FLAG antibodies. *C*, chromatographic distribution of endogenous ABIN-1, NEMO/IKKγ, and IKKβ. Cytoplasmic extracts were prepared from HEK293 treated with TNF for 120 min or left unstimulated, and subjected to chromatography on a Superdex S-200 column. Fractions were analyzed by Western blot (*WB*) by using the indicated antibodies. Molecular weight markers are indicated at the *top* of the figure.

Anti-ABIN-1 polyclonal antibodies were generated in rabbits, by using a recombinant peptide encompassing amino acids 380–636 of human ABIN-1. Other antibodies used for this study were: FLAG epitope (Sigma), A20 (BD Pharmingen), HA epitope, NEMO/IKKγ, IKKβ, IκBα, and tubulin (Santa Cruz Biotechnologies). Human TNF-α (Peprotech Inc.) was used at 2,000 units/ml.

Human ABIN-1 was amplified by PCR from a human liver c-DNA library (Clontech) and cloned into pcDNA3.1-HA, -FLAG, and -His vectors (Invitrogen) for expression in mammalian cells. A20, TAX, and ubiquitin expression vectors were gifts from G. Natoli, T. K. Jeang, and G. Courtois, respectively. NEMO/IKKγ, IKKβ, CIKS, and TRAF2 expression vectors were previously described (21, 32). All deletion mutants were prepared by conventional PCR and cloned into pcDNA3.1-HA or -FLAG vectors. Point mutants of A20 (C103S and D100A/C103S) were generated by the QuikChange Site-directed Mutagenesis kit (Stratagene), according to the manufacturer's protocol.

Yeast Two-hybrid Screening—The cDNA encoding the N-terminal part of mouse NEMO/IKKγ (amino acids 1–311) was cloned in-frame into the GAL-4 DNA-binding domain vector pGBKT7 (Clontech). The resulting plasmid pGBKT7-NEMO/IKKγ was used as bait in a yeast two-hybrid screen of a human liver cDNA library (Clontech) in *Saccharomyces cerevisiae* strain AH109. The NEMO/IKKγ deletion mutants for two-hybrid mapping were made by conventional PCR and cloned into the pGBKT7 vector.

Gel Filtration of Cellular Extracts—Gel filtration procedures were performed as previously described (42). Fractions were analyzed by Western blotting for ABIN-1, NEMO/IKKγ, and IKKβ.

In Vitro Translation and GST Pull-down Assays—*In vitro* transcription and translation were carried out with 1 μg of ABIN-1 constructs according to the TNT Quick Coupled Transcription/Translation System protocol (Promega) in the presence of [^{35}S]methionine.

GST-NEMO/IKKγ fusion protein was produced and purified as described (33). GST pull-down assays were performed by incubating an aliquot of GST-NEMO/IKKγ bound to glutathione-Sepharose beads (Amersham Biosciences) together with 10 μl of *in vitro* translated ABIN-1 protein in phosphate-buffered saline, 1% Triton X-100 buffer (including Complete Protease Inhibitor mixture (Roche)) for 2 h at 4 °C. Beads were then washed five times with the same buffer, resuspended in Laemmli buffer, and run on a SDS-polyacrylamide gel before autoradiography.

Transfection, Immunoprecipitation, and Luciferase Assay— Lipofectamine-mediated transfections were performed according to the manufacturer's instructions (Invitrogen). All transfections included supplemental empty vector to ensure that the total amount of transfected DNA was kept constant in each dish culture.

For immunoprecipitation of transfected proteins, HEK293 cells (3×10^6) were transiently transfected and 24 h after transfection cells were lysed in Triton X-100 lysis buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, and Complete Protease Inhibitor mixture). After an additional 15 min on ice, cell extracts were centrifuged for 10 min at 14,000 × *g* at 4 °C and supernatants were incubated for 4 h at 4 °C with anti-FLAG antibodies bound to agarose beads (M2, Sigma). The immunoprecipitates were washed five times with Triton X-100 lysis buffer and subjected to SDS-PAGE.

For luciferase assay, HEK293 cells (4×10^5) were seeded in 6-well plates. After 12 h cells were transfected with 0.5 μg of Ig-κB-luciferase reporter plasmid and various combinations of expression plasmids. 24 h after transfection, cells were stimulated with TNF-α for 3 h or left untreated. Cell extracts were prepared and reporter gene activity was determined via the luciferase assay system (Promega). Expression of the pRSV-β-galactosidase vector (0.2 μg) was used to normalize transfection efficiencies.

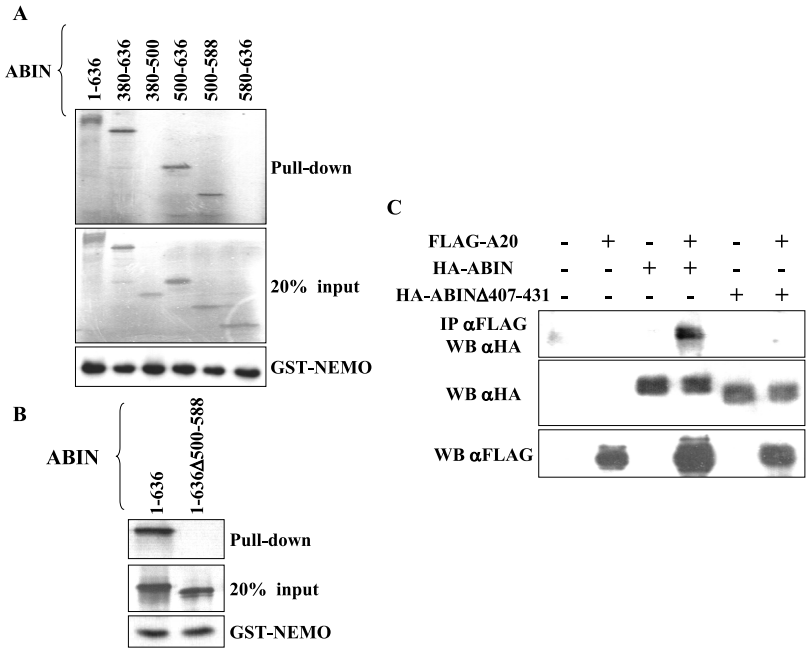

FIGURE 2. **Mapping of the NEMO/IKKγ and the A20 binding domains on ABIN-1.** *A* and *B*, GST pull-down assays: GST-NEMO/IKKγ was incubated with *in vitro* translated full-length (FL) or deletion mutants of ABIN. Aliquots of *in vitro* translated constructs and GST-NEMO/IKKγ stained by Coomassie Blue are shown. *C*, co-immunoprecipitation of FLAG-A20 with HA-ABIN or ABINΔ407–431. HEK293 cells were transfected with FLAG-A20 and either HA-ABIN or ABINΔ407–431. Cell extracts were immunoprecipitated with anti-FLAG antibodies (A20) followed by Western blot (*WB*) anti-HA (ABIN). The presence of −HA and −FLAG proteins in total extracts is shown.

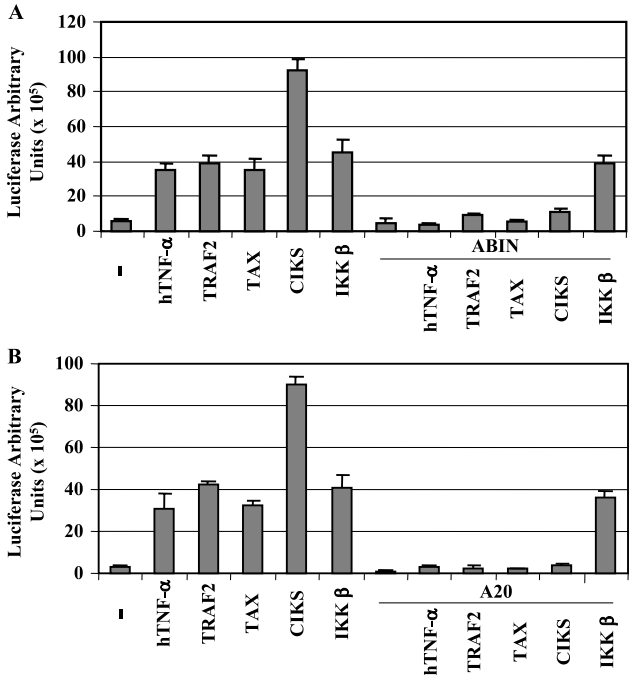

FIGURE 3. **ABIN-1 and A20 are inhibitors of NF-κB.** *A* and *B*, ABIN-1 and A20 inhibit NF-κB at level of the IKK complex. Relative reporter activity was evaluated in HEK293 cells co-transfected with the Ig-κB-luciferase plasmid and the indicated expression vectors. 24 h after transfection cells were stimulated with TNF-α for 3 h or left untreated, as indicated. Values shown in arbitrary units represent the mean ± S.D. of three experiments done in triplicate, normalized for β-galactosidase expression of a co-transfected pRSV-β-galactosidase plasmid.

In Vivo Ubiquitination and De-ubiquitination Assays— HEK293 cells (3×10^6) were co-transfected with expression vectors containing epitope-tagged ubiquitin (1 mg) and NEMO/IKKγ (200 ng), plus various constructs encoding A20 or ABIN-1 proteins. 24 h after transfection, cell lysates were prepared as above and analyzed for polyubiquitination of NEMO/IKKγ either by Western blot anti-NEMO/IKKγ (-FLAG) on total extracts or by immunoprecipitating FLAG-NEMO/IKKγ with anti-FLAG beads followed by Western blot anti-HA-ubiquitin.

ABIN-1 Small Interfering RNA (siRNA) Expression Vectors— To knockdown ABIN-1 expression, we designed double-stranded oligonucleotides containing sequences derived from the human ABIN-1 open reading frame (nucleotides 1136–1156 and 1685–1705) in forward and reverse orientations separated by a 7-base pair spacer region (caagaga) to allow the formation of the hairpin structure in the expressed siRNAs; ABINi-370: sense strand, 5′-aattcGAGGAGACCGACAAGGAGCAGcaagagaCTGCTCCTTGTCGGTCTCCTCtttttc; antisense strand, 5′-tcgagaaaaaGAGGAGACCGACAAGGAGCAGtctcttgCTGCTCCTTGTCGGTCTCCTCg; ABINi-560: sense strand, 5′-aattcCCACACCATGGCTTCGAGGACcaagagaGTCCTCGAAGCCATGGTGTGGttttc; antisense strand, 5′-tcgagaaaaaCCACACCATGGCTTCGAGGACtctcttgGTCCTCGAAGCCATGGTGTGGg. The resulting double-stranded oligonucleotides were cloned into the pcRNAi vector that we derived from the pcDNA3.1 vector (Invitrogen) by replacing the viral promoter cassette with the H1 gene promoter that is specifically recognized by RNA polymerase III. The plasmids used to knockdown A20 expression (pU6-A20i and the pU6) were a kind gift of Dr. S. Yamaoka and have been previously described (43).

RESULTS

ABIN-1 Binds to NEMO/IKKγ—The regulatory subunit of the IKK complex, NEMO/IKKγ, has an essential role in NF-κB activation. To gain insights into how NEMO/IKKγ modulates the activation of NF-κB, we screened a human liver cDNA library for NEMO/IKKγ interacting proteins, via the yeast two-hybrid system. 25 clones were identified that expressed NEMO/IKKγ-interacting proteins, including IKKα and CARMA (23). Three clones encoded for overlapping fragments of ABIN-1, a protein previously identified as an A20-binding protein that mimics the NF-κB inhibiting effects of A20 (34).

To define the region of NEMO/IKKγ that interacts with ABIN-1, we tested various deletion mutants of NEMO/IKKγ for binding to the ABIN-1 fragment (amino acids 380–636) in yeast. Data shown in Fig. 1*A* indicate that the region between amino acids 50 and 100 of NEMO/IKKγ is required for interaction with ABIN-1. The binding was confirmed in mammalian cells (Fig. 1*B*). HA-ABIN-1 was transiently co-expressed in HEK293 cells together with FLAG-NEMO/IKKγ or a NEMO/IKKγ mutant lacking the first 91 amino acids (FLAG-

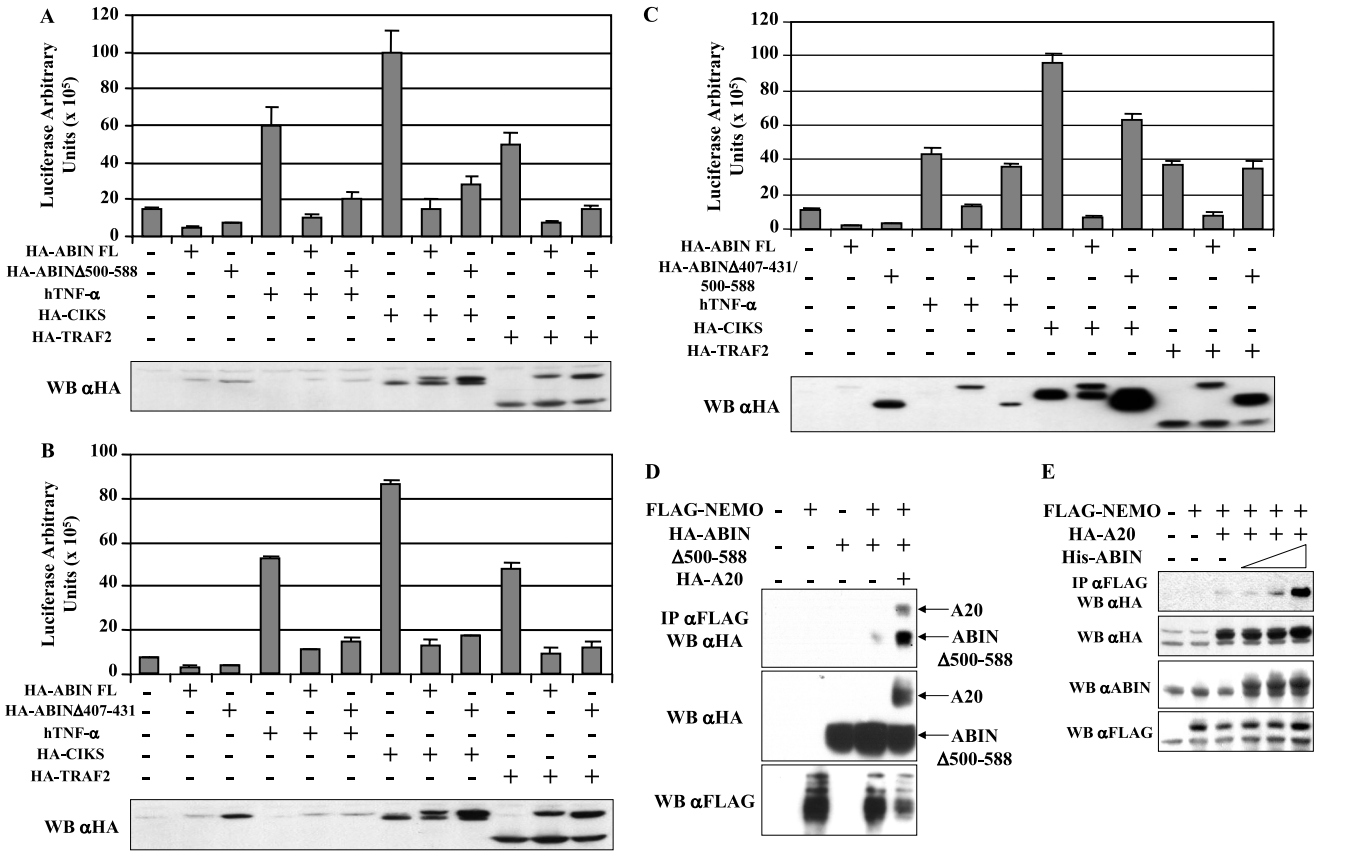

FIGURE 4. **ABIN-1 interacts with NEMO/IKKγ and A20 to inhibit NF-κB activity.** *A–C*, the deletion mutant of ABIN-1 lacking both NEMO/IKKγ- and A20-binding domains (ABINΔ407–431/Δ500–588) does not block NF-κB activation (*C*), in contrast to ABINΔ500–588 (*A*) and ABINΔ407–431 (*B*). HEK293 cells were co-transfected with the Ig-κB-luciferase reporter plasmid and the indicated combinations of expression plasmids. 24 h after transfection cells were stimulated with TNF-α for 3 h or left untreated, as indicated. Analysis was done as in Fig. 3. *Lower panels* in *A–C* show relative expression levels of each of the transfected proteins. *D*, ABIN-1 forms a complex with NEMO/IKKγ and A20. HEK293 cells were transfected with constructs encoding NEMO/IKKγ, A20, and a deletion mutant of ABIN lacking the NEMO/IKKγ-binding domain (ABINΔ500–588). Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ) and Western blotted (*WB*) anti-HA to reveal the co-precipitation of A20 and ABINΔ500–588. The presence of −HA and −FLAG proteins in total extracts is shown. *E*, ABIN-1 promotes association of A20 with NEMO/IKKγ. HEK293 cells were transiently transfected with constructs encoding FLAG-NEMO/IKKγ, HA-A20, and an increasing amount of His-ABIN. Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ) and Western blotted anti-HA to reveal the co-precipitation of A20. The presence of ABIN, NEMO/IKKγ, and A20 in the whole cell lysate is shown.

NEMOΔN91). Immunoprecipitates of FLAG-NEMO/IKKγ contained HA-ABIN-1 only if both proteins were co-expressed (Fig. 1*B*, compare *lanes 3* and *4*). In agreement with the data obtained in yeast, ABIN-1 did not co-immunoprecipitate with NEMOΔN91 (*lane 6*, Fig. 1*B*). We were unable to detect the association between endogenous NEMO/IKKγ and ABIN-1, probably because of the transient nature of the association and/or the high stringent conditions we used to perform co-immunoprecipitation experiments. However, gel filtration experiments showed that endogenous ABIN-1 was eluted from the column in the same fractions containing endogenous NEMO/IKKγ and other components of IKK complex (Fig. 1*C*).

Mapping of the NEMO/IKKγ and the A20 Binding Domains on ABIN-1—To define the domain of ABIN-1 required for its interaction with NEMO/IKKγ, we performed pull-down assays by using recombinant GST-NEMO/IKKγ and *in vitro* translated [^{35}S]ABIN-1 (Fig. 2*A*). ABIN-1 binds to GST-NEMO/IKKγ, indicating a direct interaction between the two proteins. Furthermore, amino acids 500–588 of ABIN-1 represent the minimal region that binds to NEMO/IKKγ (Fig. 2*A*, *upper panel*). To confirm that the region between amino acids 500 and 588 of ABIN-1 was responsible for interaction with NEMO/IKKγ, we generated an internal deletion mutant of ABIN-1 (Δ500–588) and evaluated its ability to interact with NEMO/IKKγ. As expected, the internal deletion of 89 amino acids from ABIN-1 abolished the interaction with NEMO/IKKγ (Fig. 2*B*). Because ABIN-1 was identified as an A20-interacting protein (35), we confirmed that the region between amino acids 407 and 431 of ABIN-1 is responsible for interaction with A20 (Fig. 2*C*).

Both ABIN-1 and A20 Inhibit NF-κB at the Level of the IKK Complex by Associating with NEMO/IKKγ—Both ABIN-1 and A20 are inhibitors of NF-κB. It has been proposed that they interfere with a RIP and TRAF2-mediated transactivation signal (34). The identification of the interaction between ABIN-1 and NEMO/IKKγ prompted us to investigate if ABIN-1 was involved in controlling NF-κB activation not only upstream but also at the level of the IKK complex. To this aim, we performed reporter assays by transfecting HEK293 cells with the Ig-κB-luciferase plasmid in the presence of ABIN-1 and various activators of NF-κB. ABIN-1 efficiently inhibited TNF-α and TRAF2-dependent activation of NF-κB (Fig. 3*A*). ABIN-1 also blocked NF-κB activation induced by proteins acting at the level of the IKK complex, such as CIKS and TAX (18–21). In contrast, ABIN-1 was not able to inhibit the IKKβ-mediated activation of NF-κB (Fig. 3*A*). Similarly, A20 inhibits NF-κB activation mediated by TNF-α or ectopic expression of TRAF2, CIKS, and TAX but not IKKβ (Fig. 3*B*). These results indicate that both ABIN-1 and A20 interfere with activation of NF-κB at the level of the IKK complex, suggesting that the association of ABIN-1 with NEMO/IKKγ could play an important role in the inhibition of NF-κB.

Because ABIN-1 interacts with both NEMO/IKKγ and A20, we tested whether the NEMO/IKKγ- and A20-binding domains of ABIN-1 were required for inhibition of NF-κB. ABIN-1 deletion mutants lacking

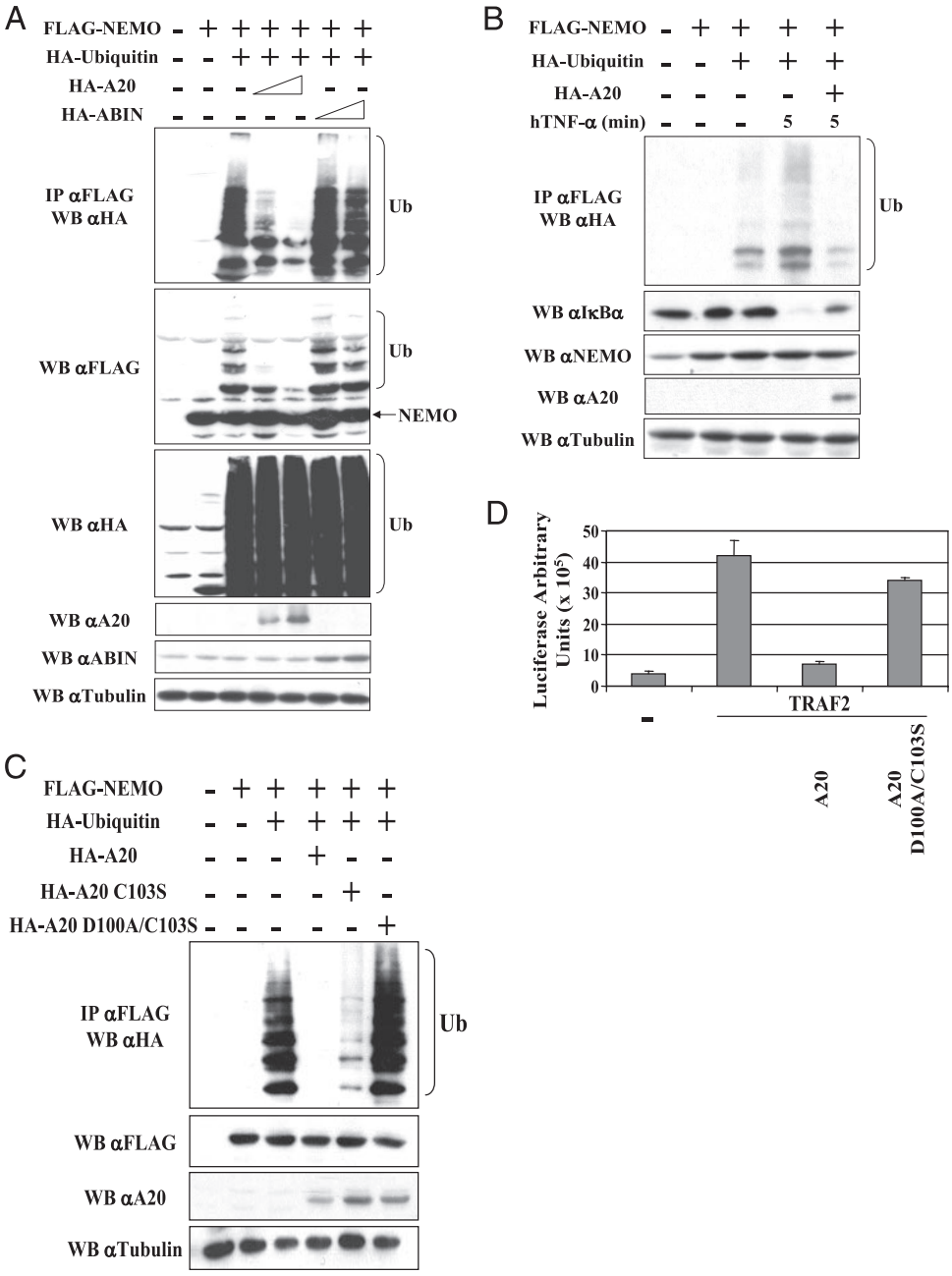

FIGURE 5. **A20 inhibits NF-κB by de-ubiquitinating NEMO/IKKγ.** *A*, A20 but not ABIN-1 de-ubiquitinates NEMO/IKKγ. HEK293 cells were transfected with FLAG-NEMO/IKKγ and HA-ubiquitin, plus increasing amounts of either HA-A20 or HA-ABIN. Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ) followed by Western blot analysis with anti-HA antibodies to reveal the polyubiquitinated forms of NEMO/IKKγ. Western blot analyses with anti-FLAG, -HA, -A20, -ABIN, and -tubulin antibodies were performed on total extracts. *B*, A20 blocks the ubiquitination of NEMO/IKKγ and the degradation of IκBα induced by TNF-α. HEK293 cells were transfected with FLAG-NEMO/IKKγ, HA-ubiquitin, and HA-A20. 24 h after transfection cells were stimulated with TNF-α for 5 min or left untreated, as indicated. Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ) and Western blotted with anti-HA antibodies. Western blots of anti-NEMO/IKKγ, -IκBα, -A20, and -tubulin are shown. *C*, a catalytically inactive form of A20 (D100A/C103S) does not de-ubiquitinate NEMO/IKKγ. Conditions were similar to those in *A*, except for the plasmids encoding HA-A20 C103S, or D100A/C103S. *D*, A20 D100A/C103S does not inhibit NF-κB activation dependent on TRAF2 in contrast to wild type A20. Reporter assay was performed by co-transfection of the Ig-κB-luciferase plasmid with combinations of TRAF2, plus A20-WT, or -D100A/C103S. Values shown in arbitrary units represent the mean ± S.D. of three experiments done in triplicate, normalized for β-galactosidase expression of a co-transfected pRSV-β-galactosidase plasmid.

either the NEMO/IKKγ binding domain (ABINΔ500–588) or the A20 binding domain (ABINΔ407–431) were still able to inhibit the activity of a NF-κB-driven luciferase reporter following different stimuli (Fig. 4, *A* and *B*). In contrast, a mutant of ABIN-1 in which both the NEMO/IKKγ- and the A20-binding domains were deleted (ABINΔ407–431/Δ500–588) lost the ability to block activation of NF-κB (Fig. 4*C*). These data were consistent with the hypothesis that ABIN-1 forms a complex with both NEMO/IKKγ and A20. To address this hypothesis, we immunoprecipitated FLAG-NEMO/IKKγ and monitored the co-precipitation of the ABIN-1 mutant lacking the NEMO/IKKγ-binding domain (HA-ABINΔ500–588) either in the presence or absence of A20 (Fig. 4*D*). ABINΔ500–588 co-immunoprecipitated with NEMO/IKKγ only in the presence of A20 (Fig. 4*D*). To further support the idea that ABIN-1 promotes association of A20 with NEMO/IKKγ, we transfected A20 and NEMO/IKKγ in the presence of an increasing amount of over-expressed ABIN-1. As expected, the amount of A20 co-immunoprecipitating with NEMO/IKKγ increased in the presence of ABIN-1 (Fig. 4*E*). Taken together, these data indicated that ABIN-1 interferes with activation of NF-κB at the level of the IKK complex, and support the idea that ABIN-1 promotes association of A20 with NEMO/IKKγ.

A20 Inhibits NF-κB by De-ubiquitinating NEMO/IKKγ—To explore the mechanism by which the interactions of both A20 and ABIN-1 with NEMO/IKKγ down-regulate NF-κB signaling, we assessed the effect of either A20 or ABIN-1 on NEMO/IKKγ ubiquitination. Transfection of FLAG-NEMO/IKKγ in the presence of HA-ubiquitin results in the polyubiquitination of NEMO/IKKγ (Fig. 5*A*, *lane 3*). Co-transfection of A20 and NEMO/IKKγ resulted in a dose-dependent disappearance of the ubiquitinated forms of NEMO/IKKγ (Fig. 5*A*, *lanes 4* and *5*). In contrast, co-transfection of ABIN-1 did not affect NEMO/IKKγ ubiquitination (Fig. 5*A*, *lanes 6* and *7*). We did not observe reduction in the overall level of ubiquitinated cellular proteins in the presence of A20, indicating that A20 does not have a global de-ubiquitinating activity in

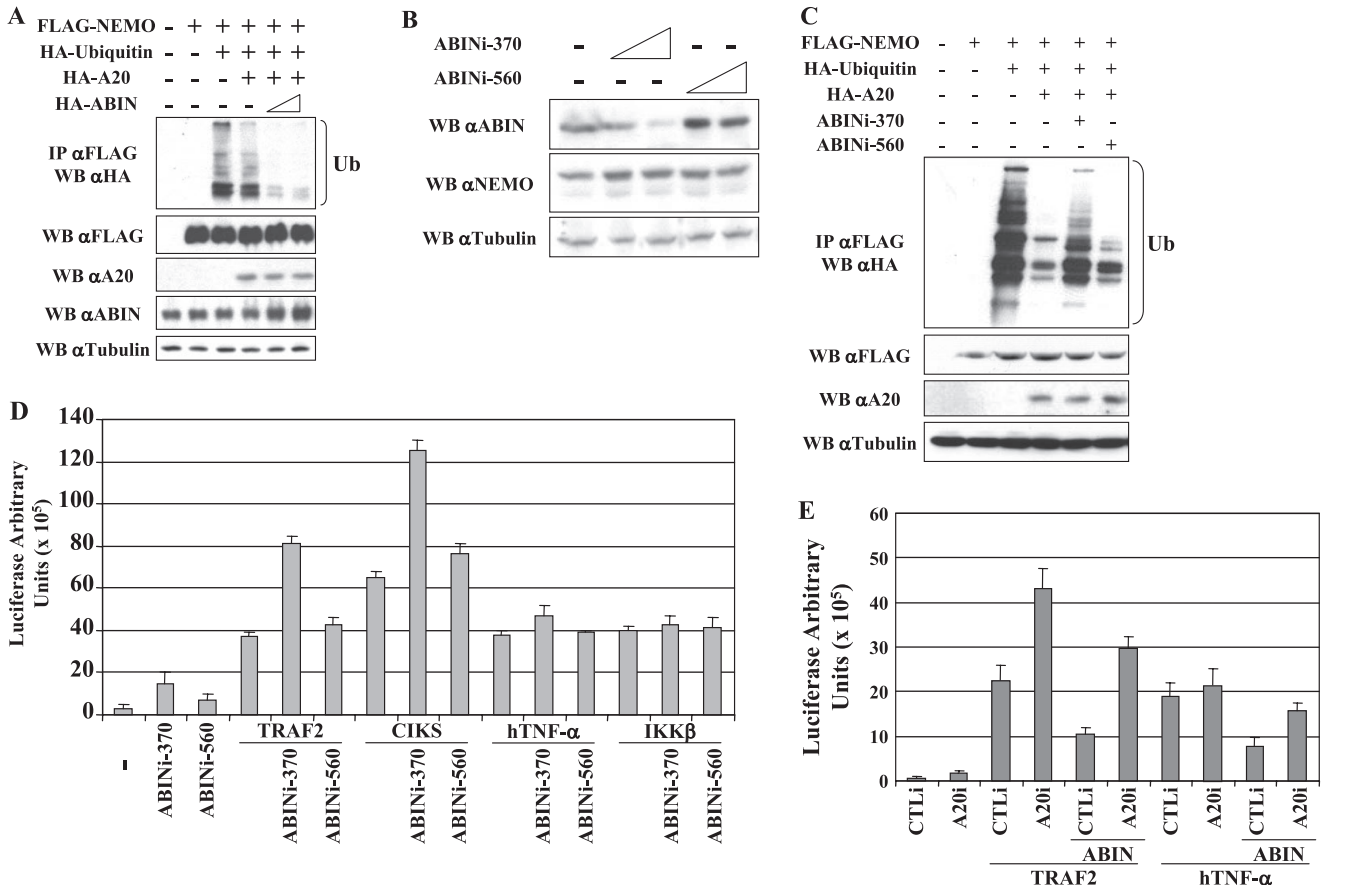

FIGURE 6. **ABIN-1 participates to the A20-dependent de-ubiquitination of NEMO/IKKγ.** *A*, ABIN-1 increases the effects of A20 on NEMO/IKKγ ubiquitination. HEK293 cells were transfected with FLAG-NEMO/IKKγ, HA-ubiquitin, suboptimal amounts of HA-A20, and increasing amounts of HA-ABIN. Cell extracts were immunoprecipitated with anti-FLAG antibodies (NEMO/IKKγ) and analyzed by Western blot for anti-HA. Western blots of anti-FLAG, -A20, -ABIN, and -tubulin are shown. *B*, RNA interference of ABIN-1. HEK293 cells were transiently transfected with siRNAs targeting ABIN (ABINi-370 or -560). ABIN expression was analyzed by immunoblotting with antibodies against ABIN. Equivalency of protein loading is shown in the tubulin and NEMO/IKKγ blots. *C*, ABIN-1 siRNAs impairs the A20-dependent de-ubiquitination of NEMO/IKKγ. HEK293 cells were co-transfected with FLAG-NEMO/IKKγ, HA-ubiquitin, and -A20, plus either ABINi-370 or -560. Cell extracts were immunoprecipitated with anti-FLAG antibodies and Western blotted with anti-HA. Western blots of anti-FLAG, -A20, and -tubulin were performed on total extracts. *D*, ABIN-1 siRNAs increase NF-κB activation by TRAF2 and CIKS. HEK293 cells were co-transfected with TRAF2, CIKS, or IKKβ, plus Ig-κB-luciferase reporter and either ABINi-370 or -570. 24 h after transfection cells were stimulated with TNF for 3 h or left untreated. Analysis was done as in Fig. 5*D*. *E*, RNA interference of A20 impairs the ABIN-1-mediated inhibition of NF-κB. Relative reporter activity was evaluated in HEK293 cells co-transfected with the Ig-κB-luciferase reporter, TRAF2, and either A20i or CTLi plasmids. 24 h after transfection cells were stimulated with TNF-α for 3 h or left untreated, as indicated. Analysis of luciferase activity was done as above.

cultured cells (Fig. 5*A*). Importantly, A20 also blocks IκBα degradation and NEMO/IKKγ ubiquitination induced by TNF-α (Fig. 5*B*). To demonstrate that the de-ubiquitinating activity of A20 was required for the observed reduction in NEMO/IKKγ ubiquitination, we generated two mutants in the OTU domain of A20, which is the domain responsible for the de-ubiquitinating activity of A20 (36). We replaced the cysteine residue of the D*XX*C motif with serine (C103S), and both the aspartic acid and the cysteine residues (D100A/C103S) with alanine and serine, respectively. The mutation C103S affected the ability of A20 to de-ubiquitinate NEMO/IKKγ compared with wild type A20, whereas the double mutant D100A/C103S resulted in the complete loss of the de-ubiquitinating activity of A20 on NEMO/IKKγ (Fig. 5*C*). As expected, the D100A/C103S mutant was not able to block the NF-κB activity induced by different stimuli, such as TRAF2 (Fig. 5*D* and data not shown).

These findings strongly suggest that NEMO/IKKγ is a target of the de-ubiquitinating activity of A20 and confirmed that the ubiquitination of NEMO/IKKγ is a crucial step in the mechanisms of NF-κB activation.

ABIN-1 Mediates the De-ubiquitinating Activity of A20 on NEMO/IKKγ—Next, we explored whether ABIN-1 was involved in the A20-dependent de-ubiquitination of NEMO/IKKγ. To this purpose, we transfected HEK293 cells with a suboptimal amount of A20 and an increasing amount of ABIN-1 and checked for NEMO/IKKγ ubiquitination. We found that ABIN-1 increases the ability of A20 to de-ubiquitinate NEMO/IKKγ (Fig. 6*A*). To demonstrate a role for ABIN-1 in the A20-mediated de-ubiquitination of NEMO/IKKγ, we generated siRNA constructs targeting ABIN-1 (ABINi-370 and i-560). Fig. 6*B* shows that the construct i-370 knocked-down ABIN-1 expression, whereas the i-560 construct did not. Then, we evaluated whether interference of ABIN-1 impairs the de-ubiquitinating activity of A20 on NEMO/IKKγ. We co-transfected HEK293 cells with FLAG-NEMO/IKKγ and HA-ubiquitin and assessed the de-ubiquitinating activity of A20 alone or in the presence of either i-370 or i-560 constructs. The A20-dependent de-ubiquitination of NEMO/IKKγ decreased only in the presence of the i-370 construct (Fig. 6*C*). The i-370 construct led to a 2-fold increase of both basal and induced (TRAF2 and CIKS) NF-κB activity compared with the empty vector or the i-560 construct, which we used as controls (Fig. 6*D*). Accordingly with the data shown in Fig. 3*A*, interference of ABIN-1 did not influence the activation of the NF-κB-dependent transfected IKKβ. Also in this case, NF-κB activity correlated with the levels of NEMO/IKKγ ubiquitination. In fact, transfected i-370 increased the ubiquitination of NEMO/IKKγ with respect to both empty vector and i-560 (data not shown). From these experiments, we concluded that reduced levels of the ABIN-1 protein affect the ability of

A20 to de-ubiquitinate NEMO/IKKγ and, consequently the A20-mediated inhibition of NF-κB. To further support the functional interplay between ABIN-1 and A20, we knocked-down A20 (43) and evaluated the ability of ABIN-1 to interfere with NF-κB activation. As shown in Fig. 6*E*, ABIN-1 requires A20 to efficiently block NF-κB activation induced by TNF and TRAF2.

DISCUSSION

In the present study, we have performed experiments in yeast, *in vitro* and in transfected cells demonstrating that ABIN-1 physically associates with NEMO/IKKγ. The functional consequence of this interaction is that overexpression of ABIN-1 blocks not only activation of NF-κB upstream of the IKK complex, but also NF-κB activation mediated by proteins directly contacting the IKK complex, such as CIKS and TAX. Indeed, activation of NF-κB mediated by overexpression of IKKβ, which may be considered functionally downstream of NEMO/IKKγ, is not affected by ABIN-1. ABIN-1 has been identified as an A20-binding protein and its overexpression mimics the inhibitory effect of A20, suggesting that ABIN-1, at least partially, mediates the inhibitory function of A20. A20 is a TNF-α responsive gene that inhibits NF-κB-dependent gene transcription in response to TNF-α and other stimuli (37–39). Given that A20 expression itself is regulated by NF-κB, it is possible that A20 and ABIN-1 participate in a negative feedback regulation of NF-κB activation (40, 41). However, the mechanism by which ABIN-1 participates in this process is not clear. We provide evidence that ABIN-1 and A20 co-operate to promote de-ubiquitination of NEMO/IKKγ, which results in functional inactivation of NF-κB. In fact, recent studies have indicated ubiquitination as a key event in the regulation of NF-κB activation. For example, A20 modifies the ubiquitination profile of RIP in a two-step model, by removing the Lys^{63}-linked ubiquitin chain and by the subsequent ligation of the Lys^{48}-linked ubiquitin chain (30). Similarly, A20 inhibits Toll-like receptor signaling by removing the Lys^{63}-linked ubiquitin chain from TRAF6 (27). In this context our data demonstrate that NEMO/IKKγ is an additional target of the de-ubiquitinating activity of A20. Currently, it is not clear if A20 directly de-ubiquitinates NEMO/IKKγ, or it affects the activity of other protein(s) regulating NEMO/IKKγ ubiquitination. However, we have now shown that in cells knocked-down for ABIN-1, we observed a decrease in the ability of A20 to de-ubiquitinate NEMO/IKKγ, and that in cells knocked-down for A20, the inhibitory function of ABIN-1 is impaired. Altogether our data strongly suggest that ABIN-1 functionally connects A20 and NEMO/IKKγ.

In summary, we have identified a previously not reported association between ABIN-1 and NEMO/IKKγ and we provide evidence that ABIN-1 co-operates with A20 in inhibiting NF-κB at the level of the IKK complex. In addition, we propose that this association could target A20 on NEMO/IKKγ and interfere with NEMO/IKKγ ubiquitination, to negatively regulate NF-κB activation.

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A novel insertional mutation in the prion protein gene: clinical and bio-molecular findings

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A novel insertional mutation in the prion protein gene: clinical and bio-molecular findings

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ABSTRACT

A young man, presenting with early onset of personality and behavioural changes followed by slowly progressive cognitive impairment associated with marked bi-parietal cerebral atrophy, was found to carry a novel seven extra-repeat insertional mutation in the prion protein gene (*PRNP*). In vitro, the mutated recombinant prion protein (PrP) showed biochemical properties that were consistent with pathological PrP variants. Our results further underline the heterogeneity of neurological pictures associated with insertional mutations of *PRNP*, indicating the diagnostic difficulties of sporadic cases with early-onset atypical dementia.

Prion diseases are a heterogeneous group of neurodegenerative disorders, including sporadic, genetic and acquired forms. The causative agent consists of conformationally altered forms of a host-encoded glycoprotein (PrP^{C}), termed PrP^{Sc}.[1] The genetic forms of prion diseases are associated with point or insertional mutations in the PrP gene (*PRNP*). PrP^{C} carries one nonapeptide (R1) and four octapeptides (R2, R2, R3, R4) between codons 51 and 91, which have the same amino-acid sequence but can be distinguished by variations in their DNA sequence. Insertional mutations consist of one, two, four, five, six, seven, eight or nine extra octapeptide repeats in *PRNP*. Although patients with one, two or four extra repeats have typical Creutzfeldt–Jakob disease (CJD), the clinico-pathological features of patients with five, six, seven, eight and nine extra repeats are reminiscent of Gerstmann–Sträussler–Scheinker disease or atypical dementia.[2 3]

Here, we report a patient with an early onset, slowly progressive disease, manifesting personality and behavioural changes followed by atypical dementia, carrying a novel seven-octarepeat insertional mutation in *PRNP*. The results obtained *in vitro* on the mutant recombinant protein are consistent with previously reported data about the effects of the insertional mutations over the biochemical properties of the protein.[4]

PATIENT AND METHODS

Patient history

A 27-year-old man presented with slowly progressive behavioural changes and cognitive impairment, without familial history of dementia or psychosis. The pre-clinical history revealed that, as a teenager, he was an active, competitive and well-adapted person, even a successful student. At 19 years of age, the disease became manifest with loss of social interests, apathy, emotional indifference and progressive isolation at home. A few years later, learning difficulties became prominent, mostly dependent on attention deficit. Hallucinations and/or delirium were not reported. During this early phase, the diagnosis of psychosis or depression was formulated and the patient was treated with neuroleptics and anti-depressive drugs, without any efficacy. In the past 2 years before our observation, he developed apraxia.

Our neurological evaluation revealed visuo-spatial and constructional defects, ideomotor apraxia, mild left–right confusion, dyscalculia and bilateral cortical dysestesia, although no ataxia, pyramidal or extrapyramidal signs were present.

Extensive viral, metabolic, endocrine and immunological investigations were unrevealing. Cerebrospinal fluid analysis was normal, including determination of 14.3.3 and tau proteins. Electroencephalographic (EEG) studies showed frequent diffuse and bilateral paroxysmal slow waves, prevailing posteriorly. Magnetic resonance imaging (MRI) and positron emission tomography (PET) disclosed diffuse cortical atrophy and reduction of 18-Fluoro-2-Deoxy-D-glucose (FDG) metabolism, mostly in the parietal lobes and particularly in the posterior parietal gyrus (fig 1A, B).

Genetic analysis

Sequence analysis of full-length coding region of *PRNP*, of microtubule-associated protein Tau (*MAPT*), and of exons 16 and 17 of amyloid beta precursor protein (*AβPP*) was performed.

More than 1 year later, the patient received further genetic counselling at another institution, and his case was briefly described.[5]

Peripheral blood leukocyte (PBL) analysis

Peripheral blood leukocytes (PBLs) from a control and the patient were purified by centrifugation and lysed in Laemmli buffer. Aliquots (40 μl) of supernatant were analysed by Western blot using monoclonal antibodies 3B5 (gift of Prof. G. Hunsmann, Göttingen, Germany—epitope at residues 79–92 of human PrP)[6] and 3F4 (Dako, Carpinteria, CA; epitope at residues 109–112 of human PrP).

Cloning of wild-type PrP and mutated recombinant PrP with seven extra repeats

Total RNA was isolated from the patient's PBLs using the Chomczynski and Sacchi method.[7] Primers to amplify wild-type PrP (WT-PrP) and mutated recombinant PrP with seven extra repeats

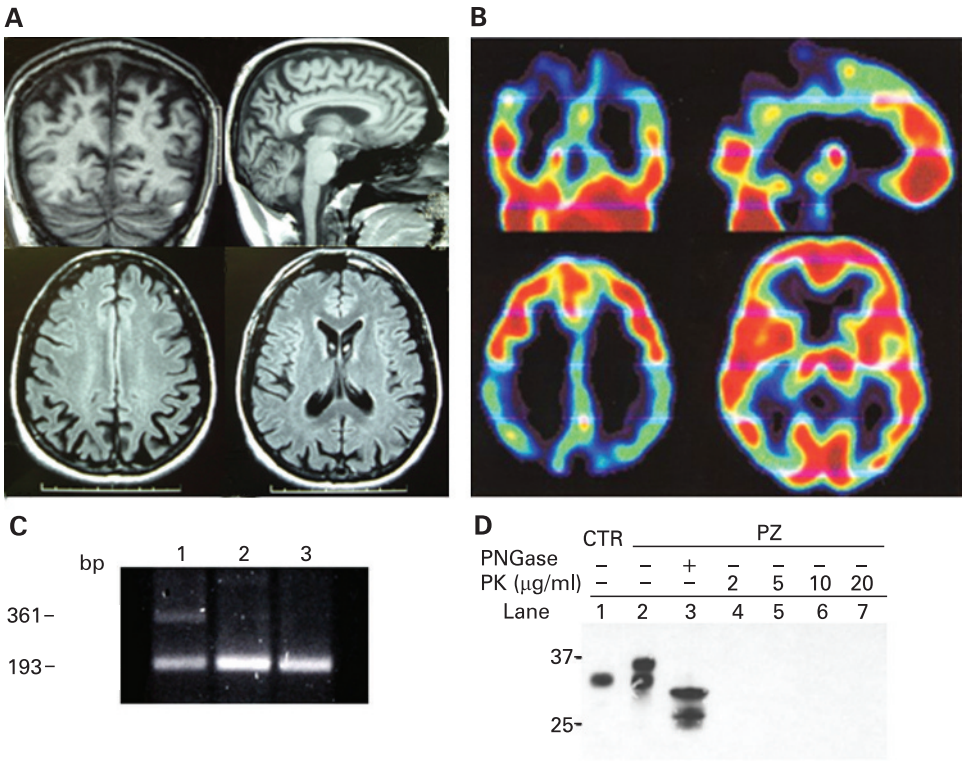

Figure 1 Brain atrophy and reduction of FDG/metabolism marked in parietal convexity bilaterally at MRI (A) and PET/FDG (B). Ethidium bromide-stained fragments of the PCR-amplified repeat region (F4-R4 primers) of *PRNP* (C). The parents (lanes *2,3*) show a single band of 193 bp. The patient (lane *1*) shows an additional fragment of 371 bp derived from the allele containing the insertional mutation. In (D), PrP expression into peripheral blood leucocytes before and after proteinase K (PK) digestion (2, 5 and 10 μg/ml) and deglycosylation (PNGase) is seen. Compared with the normal control (lane *1*), the patient with the insertional mutation (lane *2*) shows an additional band of ~39 kd before deglycosylation and ~32 kd after deglycosylation (lane *3*), which corresponds to full-length mutated recombinant PrP with seven extra repeats (M7-PrP). After PK incubation, control and M7-PrP are completely digested (lanes *4–7*). Western blot analysis (3F4 antibody, 1:50,000).

(M7-PrP) were designed on the basis of the PrP cDNA sequence (GenBank accession number BC022532): forward, 5′-CCGGAATTCGCCGCCATGGCGAACCTTGGCTGC-3′; reverse, 5′-TCTTCTAGATCCCACTATCAGGAAGATGAGG-3′.The amplified bands were cloned in the p-CMV vector for expression in mammalian cells. Cloned cDNA fragments were analysed by automatic DNA sequencing.

The detergent solubility and the proteinase K (PK) resistance assays were carried out on HEK-293 transfected cells, as previously described,[3] with slight modifications (ie, PK digestion: 5, 10 and 20 μg/ml, 37°C, 30 minutes).

RESULTS

Genetic analysis of the proband disclosed the presence of an insertional mutation consisting of seven extra repeats in *PRNP* (fig 1C). The polymorphism at codon 129 was methionine/methionine. According to Goldfarb's nomenclature,[8] the repeats of the mutant allele were arranged as follows: R1-R2-R2-R3-R2-R2-R3g-R2-R2-R2-R3-R4. Although the differences in nucleotides did not modify the amino-acid sequence of the octapeptides, DNA sequencing established that our patient carried a new *PRNP* insertion of 168 bp. This mutation was not present in his parents (study of the haplotypes excluded a paternity error). No mutations were found in *AβPP* and *MAPT* genes.

Western blot on the patient's PBLs revealed the expression of both mutant M7-PrP and WT-PrP (fig 1D). Both proteins were PK-sensitive and detergent-soluble.

When expressed in HEK-293 cells, M7-PrP revealed abnormal properties, showing PK resistance up to an enzyme concentration of 10 μg/ml (fig 2A) and detergent insolubility (fig 2B). PK digestion produced no shift in molecular weight, but increased the amount of a low molecular weight ~11 kDa fragment, which was already present in PK untreated samples. Although WT-PrP was exclusively detected in a detergent-soluble fraction, the ~11 kDa fragment and about 50% of the full-length M7-PrP were detergent insoluble.

WT- and M7-PrP disclosed a different glycosylation pattern, with a significant increase of the non-glycosylated and mono-glycosylated immature forms in M7-PrP (fig 2C). This result is suggestive of an altered folding of M7-PrP. To further support the finding that M7-PrP does not fold properly, we took advantage of the knowledge that unfolded and misfolded proteins are retro-translocated from the lumen of the ER to the cytosol and degraded through a proteosome-dependent mechanism, and checked the glycosylation pattern of WT- and M7-PrP after blocking the proteosome activity. Figure 2D shows an increase of the non-glycosylated form only in M7-PrP, following inhibition of proteosome by Calpain Inhibitor-I.

DISCUSSION

Here, we report a novel large insertional mutation in *PRNP* associated with early-onset and slowly progressive encephalopathy, leading to focal brain atrophy. To date, it represents the first reported *de novo* extra-repeat insertional mutation in *PRNP*. The likeliest genetic mechanism proposed for this spontaneous mutation is (multi-stage) replication strand slippage,[9 10] which probably occurred spontaneously in the parents' spermatogenesis/oogenesis—that is, during the DNA replication process, the daughter strand experiences slippage facilitated by complementarity to an earlier template repeat. Repeats, by their nature, are still complementary when misaligned by an integral multiple of 24 bp. Exonuclease editing 3′ to 5′ is available to remove mismatches. Partial editing produces chimeric repeats. When replication resumes with slipped complementarity in place, the result is extra repeats. Longer complex repeats require multiple rounds of stalling, slipping, editing and resuming replication, until finally replication run-out occurs. The imbalance of reciprocal recombinants, complexity of some variants and

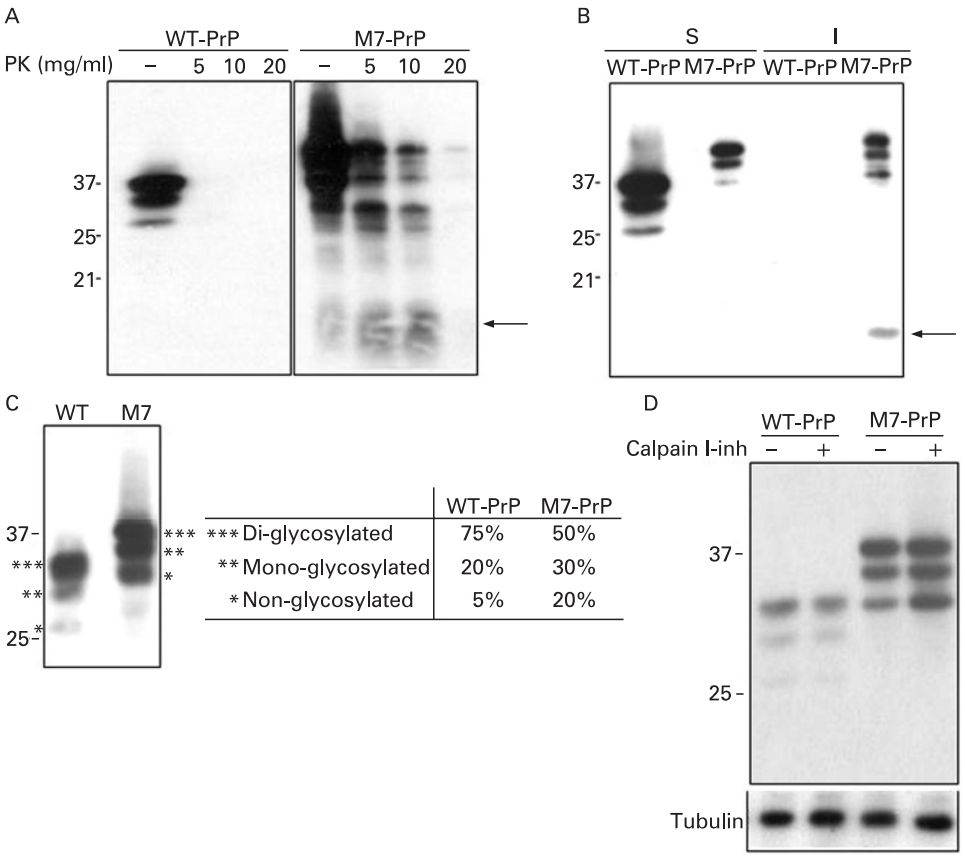

Figure 2 *In vitro* studies of WT- and M7-PrP and abnormal properties of recombinant M7-PrP. (A) Sensitivity of wild-type (WT)- and mutated recombinant PrP with seven extra repeats (M7-PrP) to limited proteolysis. WT-PrP is completely degradated by proteinase K (PK), whereas M7-PrP is partially resistant up to a PK concentration of 10 μg/ml. The PK digestion leads to an increase in the low molecular weight (~11 KDa) fragment (arrow) already evident in the absence of PK. (B) Western blot analysis of detergent-soluble (S) and detergent-insoluble (I) fractions of both WT- and M7-PrP. The WT-PrP is exclusively found in the soluble fraction. Conversely, the 11 kDa PrP fragment (arrow) and about 50% of the full-length M7-PrP are present in the insoluble fraction, with relatively higher representation of less glycosylated isofoms. (3B5 antibody, 1:50,000 (identical results with 3F4 antibody)). A different pattern of glycosylation between WT- and M7-PrP is evident (C), due to increased non- and mono-glycosylated M7-PrP isoforms, as confirmed by densitometric analysis. After inhibition of proteasome with Calpain inhibitor-I (6 h), there is a more evident increase of M7-PrP—in particular, the non-glycosylated isoform (D). (3B5 antibody, 1:50,000 (identical results with 3F4 antibody)).

generational stability are implausibly implemented via unequal recombination.

Four kindreds with seven extra-repeat mutations have been described so far,[8 11 12] all presenting a dominant pattern of inheritance and phenotypically distinct from the present case. In our case, as in all cases reported with a similar insertion, the mutation was in heterozygosis and in frame with methionine at codon 129 of *PRNP*. As previously reported in other octapeptide repeat insertions,[13] here it is confirmed that the 129-methionine/methionine polymorphism is associated with early onset of disease, but not with the phenotype of the disease and the rate of clinical progression.

We lack neuropathological study of our patient; however, all the data available in the literature about other kindreds with seven extra repeats of *PRNP* insertional mutation indicate the pathogenicity of this genetic defect, with the neuropathological hallmark lesions of prion diseases and transmission of the spongiform encephalopathy into primates.[8]

Our *in vitro* experiments support the idea that the *PRNP* mutation of our patient had a causal role. The mutated recombinant prion protein (M7-PrP) that was ectopically expressed in HEK-293 cells showed partial PK resistance and detergent insolubility, which are typical characteristics of PrP^{Sc}. It is noteworthy that PK resistance was present even at higher PK concentration and longer digestion time than previously reported.[4 14 15] Further support to the abnormal properties of M7-PrP came from the finding of a low molecular weight fragment (~11 kDa) reminiscent of GSS amyloid protein,[16] that increased after PK digestion and was detergent insoluble.

The tendency to misfolding of the recombinant M7-PrP is evoked by its altered glycosylation pattern, which suggests a build-up in the ER and an inefficient transport to the cell surface.[17] This can be explained taking into account that membrane and secreted proteins are submitted to a strict control that is aimed at preventing the transport of misfolded proteins from the ER to the cellular surface by means of the retro-translocation in the cytosol and degradation through the proteasome. Further support of this hypothesis came from the observation that inhibition of proteasomes resulted in an increase in the non-glycosylated immature glycoform of mutated recombinant PrP.

From a clinical point of view, our case showed some similarities with other patients, reported in the literature, carrying seven extra-repeats of insertional mutation, such as cognitive decline, long duration of disease and apraxia.[8 11 18 19] However, it is unique because of early onset with personality changes and autistic-like behaviour, slow progressive course, a pattern of focal dementia correlated to marked parietal atrophy and absence of cerebellar ataxia.

This study highlights the importance of searching the aetiology of early-onset sporadic dementia presenting with psychiatric features by screening for mutations of the *PRNP*, also considering some similarities with variant CJD.[20]

Competing interests: None declared.

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The neutrophil gelatinase-associated lipocalin (NGAL), a NF-κB-regulated gene, is a survival factor for thyroid neoplastic cells

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NF-κB is constitutively activated in primary human thyroid tumors, particularly in those of anaplastic type. The inhibition of NF-κB activity in the human anaplastic thyroid carcinoma cell line, FRO, leads to an increased susceptibility to chemotherapeutic drug-induced apoptosis and to the blockage of their ability to form tumors in nude mice. To identify NF-κB target genes involved in thyroid cancer, we analyzed the secretome of conditioned media from parental and NF-κB-null FRO cells. Proteomic analysis revealed that the neutrophil gelatinase-associated lipocalin (NGAL), a protein involved in inflammatory and immune responses, is secreted by FRO cells whereas its expression is strongly reduced in the NF-κB-null FRO cells. NGAL is highly expressed in human thyroid carcinomas, and knocking down its expression blocks the ability of FRO cells to grow in soft agar and form tumors in nude mice. These effects are reverted by the addition of either recombinant NGAL or FRO conditioned medium. In addition, we show that the prosurvival activity of NGAL is mediated by its ability to bind and transport iron inside the cells. Our data suggest that NF-κB contributes to thyroid tumor cell survival by controlling iron uptake via NGAL.

Cancer is a multistep process during which cells undergo alterations of their normal functions that progressively lead to the genesis of a tumor. Among the most significant changes, cancer cells begin to secrete, into the tumor microenvironment, a number of factors that stimulate their own growth/survival and mediate angiogenesis and metastasis. During the last years, the role of NF-κB in the pathogenesis of human cancer has strongly emerged. In particular, because it is now generally accepted that chronic inflammation contributes to the genesis of many solid tumors, such as gastric, colon, or hepatic carcinomas, it has been recently shown that activation of NF-κB by the classical IKKβ-dependent pathway is a crucial mediator of inflammation-induced tumor growth and progression in animal models of inflammation-associated cancer (1, 2). This is not particularly surprising given that NF-κB controls expression of a number of proinflammatory factors (cytokines, chemokines ,and growth factors), secreted by cancer cells in the tumor microenvironment, that substantially contribute to tumor development (3, 4). Understanding the molecular mechanism by which these factors play their role in cancer could help in the comprehension of the role of NF-κB in inflammation-related cancer and could open innovative perspectives in the treatment of tumors.

We have shown that NF-κB is strongly activated in human anaplastic thyroid carcinomas (ATCs) (5). To study the role of NF-κB in thyroid cancer, we inhibited its function by stably transfecting FRO cells (derived from a human ATC) with a super-repressor form of IκBα (IκBαM). As a result, FRO IκBαM cells lost their oncogenic potential mainly from an increased susceptibility to drug-induced apoptosis (5).

The neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2, is a member of a large family of lipocalins, a group of small extracellular proteins with great functional diversity (6). It is released from neutrophil granules as a 25-kDa monomer, a 46-kDa disulfide-linked homodimer, and a 135-kDa disulfide-linked heterodimer with a matrix metalloproteinase-9 (MMP-9) (7). NGAL is thought to be an acute phase protein (8) whose expression is up-regulated in human epithelial cells under different inflammatory conditions, such as inflammatory bowel disease (9). Elevated NGAL expression has also been shown in different human tumors including breast (10), lung (11), colon (9), ovary (12), and pancreas (13) carcinomas. However, the precise role of NGAL has not been well defined. Several studies have suggested that NGAL is a potent bacteriostatic agent that has siderophore-mediated sequestering of iron (14, 15), and that it is capable of protecting MMP-9 from autodegradation, thereby favoring the metastatic potential of cancer cells (16). As a secreted binding protein, it has been reported that the murine ortholog, 24p3, plays a crucial role in IL-3 deprivation-induced apoptosis by regulating intracellular iron delivery, very likely after interaction with a 24p3 receptor (17). Indeed, the human NGAL protects A459 and MCF7 cells from apoptosis induced by PDK1 inhibitors (18). Last, NGAL induces cell proliferation by promoting the iron-dependent metabolism of nucleotides for DNA synthesis (19).

The cellular system FRO/FRO IκBαM represents an excellent model for the identification of NF-κB-regulated factors that, when secreted in the extracellular milieu, could play a role in thyroid cancer. Thus, we used a different proteomic approach to analyze the pattern of expression of secreted proteins from conditioned medium of parental FRO cells and FRO IκBαM clones. One of the proteins that showed a marked decrease of expression in FRO IκBαM cells was NGAL. We show that knocking down NGAL expression blocks the ability of FRO cells to form colonies in soft agar and tumors in nude

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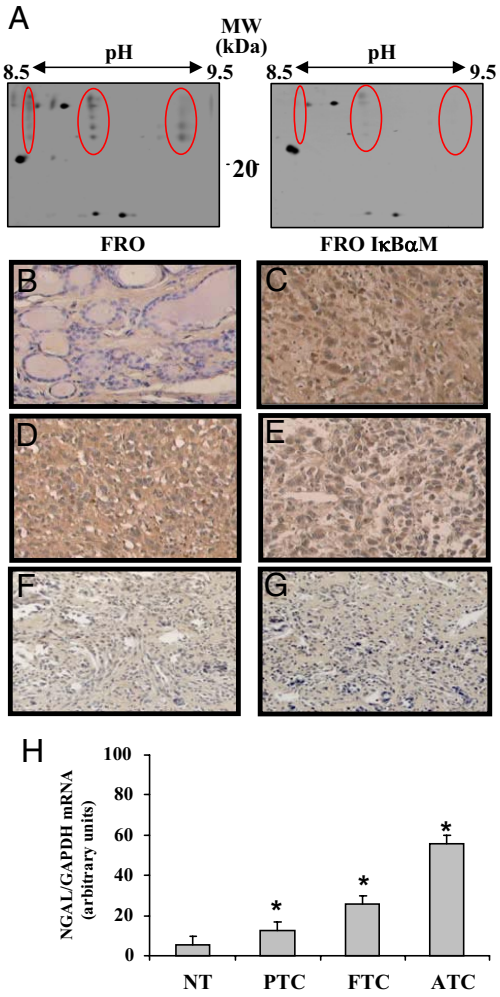

Fig. 1. Analysis of NGAL expression. Two-dimensional gel electrophoresis showing expression of NGAL in the conditioned medium of FRO IκBαM cells, as compared with parental counterpart FRO (*A*). Localization of NGAL *in situ* was determined by immunohistochemistry in sections from normal thyroid tissue (*B*) and three different ATCs (*C–E*). Lack of specific tissue staining was seen when either secondary Abs (*F*) or blocked Abs (*G*) were used. Magnification: ×400. (*H*) qRT-PCR analysis of NGAL mRNA levels in primary human cancers. NT, normal thyroid ($n = 2$); PTC, papillary thyroid carcinoma ($n = 3$); FTC, follicular thyroid carcinoma ($n = 3$); ATC, anaplastic thyroid carcinoma ($n = 3$). *, $P < 0.0005$.

mice and, therefore, increases their susceptibility to apoptosis. In addition, we show that the prosurvival activity of NGAL is mediated by its ability to bind and transport iron inside the cells.

Results

Differential Proteomic Analysis of Conditioned Media from FRO and FRO IκBαM Cell Lines. To identify factors secreted by thyroid cancer cells under transcriptional control of NF-κB, we analyzed the secretome of partially purified conditioned media from FRO and FRO IκBαM cell lines. Among the proteins whose expression decreased in the extracellular medium of FRO IκBαM cells, as compared with FRO, at least three groups were examined because of their strong reduction (Fig. 1 *A*, circled spots). The MALDI-TOF peptide mass fingerprint analysis identified all spots as NGAL, whose modifications after translation account for the multiple species that show variable molecular weight and pI values in the 2-DE gel.

Northern and Western blot assays confirmed the decreased expression of NGAL in FRO IκBαM cells, as compared with parental FRO cells [supporting information (SI) Fig. S1 *A–C*]. To confirm that NF-κB was controlling NGAL expression, we knocked down p65 expression in FRO cells by using siRNA and detected a decreased NGAL expression by Western blot analysis(Fig. S1*D*). In addition, WT and $p65^{-/-}$ mouse embryonic fibroblasts (MEF) were treated with IL1β or IL17, and the NGAL expression was detected by Northern and Western blot assays (Fig. S1 *E* and F). No induction of NGAL was detected in absence of p65. Reexpression of p65 in $p65^{-/-}$ MEF restored NGAL expression (Fig. S1*G*).

It was demonstrated that NF-κB controls NGAL expression via the inducible cofactor IκBζ (20, 21). We then investigated, by using chromatin immunoprecipitation, whether, in FRO cells, IκBζ was also bound to the NGAL promoter. As shown in Fig. S2, IκBζ was bound to the NGAL promoter together with the p50 and the p52 subunits of NF-κB.

Immunohistochemical Analysis of NGAL Expression in Normal and Pathological Human Thyroid Specimens. To verify the expression of NGAL in human primary thyroid cancer, we analyzed normal thyroid specimen and specimen from different thyroid carcinomas by immunostaining. As shown in Table 1 and Fig. 1 *B–G*, although NGAL expression was not detected in normal thyroid, NGAL immunostaining was positive for papillary, follicular and ATC. The levels of NGAL expression increased with the malignant phenotype of tumors, reaching the highest intensity in the anaplastic carcinomas (Table 1 and Fig. 1 *B–G*), in parallel with NF-κB basal activity in the same types of thyroid tumors (5). Real time PCR analysis confirmed the increased expression on the NGAL mRNA in primary human thyroid cancer (Fig. 1*H*).

Inhibition of NGAL Expression Leads to the Blockage of Tumorigenicity in FRO Cells. To study the role of NGAL in thyroid cancer, we blocked its expression in FRO cells by stable transfection of a siRNA targeting NGAL. Fig. 2*A* shows the levels of NGAL protein in two of the clones analyzed (indicated as siRNA NGAL 2/2 and 2/6) in FRO cells, either transfected with the empty vector (FRO Neo cells) or transfected with a control siRNA

Table 1. Immunohistochemical analysis of NGAL expression in normal and pathological human thyroid tissues

Histological type of thyroid samples	Number of total cases analyzed	Number of positive cases/ Number of total cases analyzed	NGAL staining score			
			0+	1+	2+	3+
Normal thyroid	3	0/3	3			
Papillary carcinoma	14	9/14	5	4	3	2
Follicular carcinoma	8	5/8	3		2	3
Anaplastic carcinoma	7	6/7	1		2	4

Human specimens were stained with anti-NGAL polyclonal antibodies. The percentage of malignant cells stained was scored from 0 to 3: 0, no positive cells; 1+, <10% of positive cells; 2+, 11–60% of positive cells; 3+, 61–100% of positive cells.

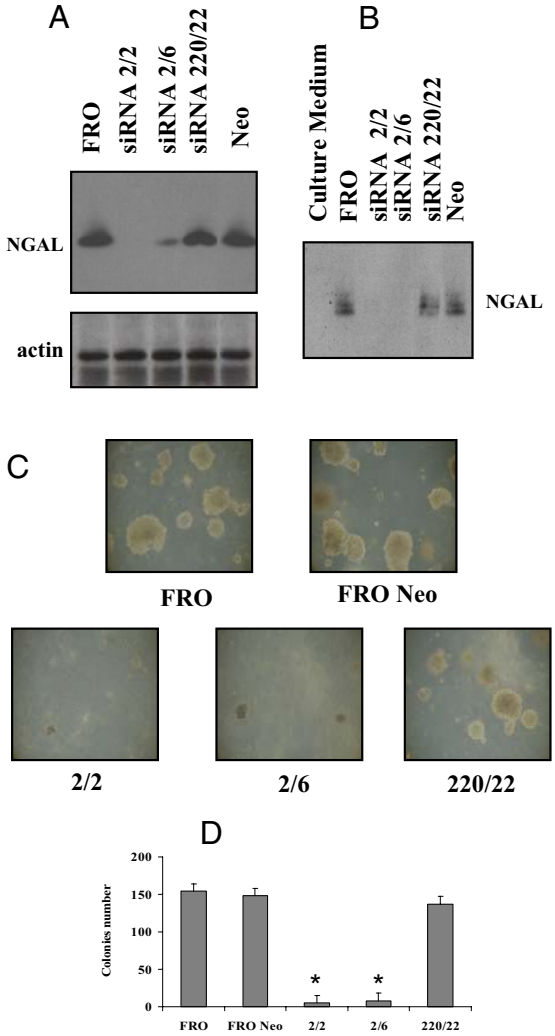

Fig. 2. Inhibition of NGAL expression by siRNA and *in vitro* oncogenic activity of the siRNA clones. The expression of NGAL in FRO cells, stably transfected with either siRNA plasmids (2/2, 2/6), control siRNA plasmid (220/22), or empty vector (Neo), was determined by Western blot analysis on total cell lysates (*A*) and on conditioned media (*B*). (*C*) Colony formation assay. Colonies >50 cells were scored after 2 weeks incubation at 37°C (*D*). Magnification: ×200. *, $P < 0.0001$ (2/2 and 2/6 vs. FRO, FRO-Neo, and 220/22).

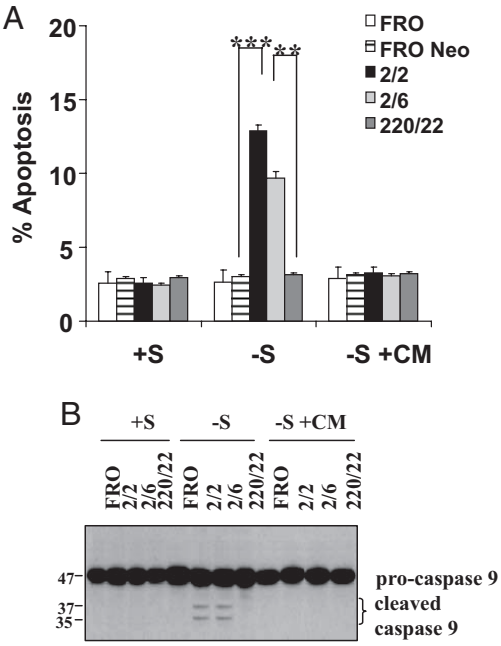

Fig. 3. NGAL protects FRO cells from apoptosis induced by serum-withdrawal. 2.5×10^5 cells per well were seeded in 6-well culture plates and grown either in the presence of serum (+*S*), in the absence of serum (−*S*), or in serum-free conditioned medium from FRO cells (−*S* + *CM*). Cell death was assessed by propidium iodide staining (*A*) and by Western blot analysis (*B*). Results were mean ± SD of at least three separate experiments. **, $P < 0.0005$; ***, $P < 0.0001$.

(indicated as siRNA NGAL 220/22). Fig. 2*B* shows the levels of secreted NGAL. These clones were tested for their ability to grow in semisolid medium and to form tumors in nude mice. In *in vitro* experiments, whereas FRO, FRO Neo, and 220/22 cells gave rise to foci of transformation, 2/2 and 2/6 clones did not form any colonies (Fig. 2 *C* and *D*). In *in vivo* assays, the injection of parental FRO or 220/22 cells into nude mice induced tumor formation in 12 of 12 mice, whereas the injection of 2/2 or 2/6 cells induced tumor formation in 1 of 6 and 4 of 6 mice, respectively (Table 2). The tumors developed from 2/2 and 2/6 cells were 4-fold and 2-fold smaller than those formed after injection of parental cells, respectively (Table 2). Notably, 2 of 4 tumors from 2/6 cells showed NGAL staining after immunohistochemical analysis, indicating that the expression of the siRNA plasmid was lost. We also blocked NF-κB in FRO cells by transient overexpression of IκBαM. In the absence of functional NF-κB, FRO cells did not form colonies in soft agar. Reexpression of NGAL partially rescued the ability of FRO cells to form colonies in soft agar (Fig. S3 *A–C*).

NGAL Is a Survival Factor in FRO Cells. We analyzed the response of parental FRO cells and NGAL siRNA clones to serum withdrawal-induced apoptosis. All clones grown in the presence of serum showed 2–3% apoptosis, as assessed by propidium iodide staining (Fig. 3*A*). Serum deprivation did not affect the survival of FRO, FRO Neo, and 220/22 clones but induced a significant cell death (10–15%) in 2/2 and 2/6 clones (Fig. 3*A*). This effect was reverted by the addition of the conditioned medium of either

Table 2. *In vivo* tumor growth induced by FRO Neo cells and FRO siRNA NGAL clones

Cell type	Tumor incidence	Tumor volume average, cm^3	Tumor weight average, g
FRO	6/6	0.37 ± 0.04	0.24 ± 0.05
FRO siRNA NGAL 2/2	1/6	0.01***	0.05***
FRO siRNA NGAL 2/6	4/6	0.16 ± 0.06**	0.11 ± 0.02**
FRO siRNA NGAL 220/22	6/6	0.33 ± 0.02	0.21 ± 0.03

2×10^7 cells were injected s. c. on a flank of each 6-week-old nude mouse. Tumor weight, diameter, and volume values were measured and determined as described in the *SI Text*. Two of four tumors developed by mice injected with FRO siRNA 2/6 cells showed partial NGAL staining after immunohistochemical analysis. ***, $P < 0.0001$ (FRO 2/2 vs. FRO); **, $P < 0.001$ (FRO 2/6 vs. FRO).

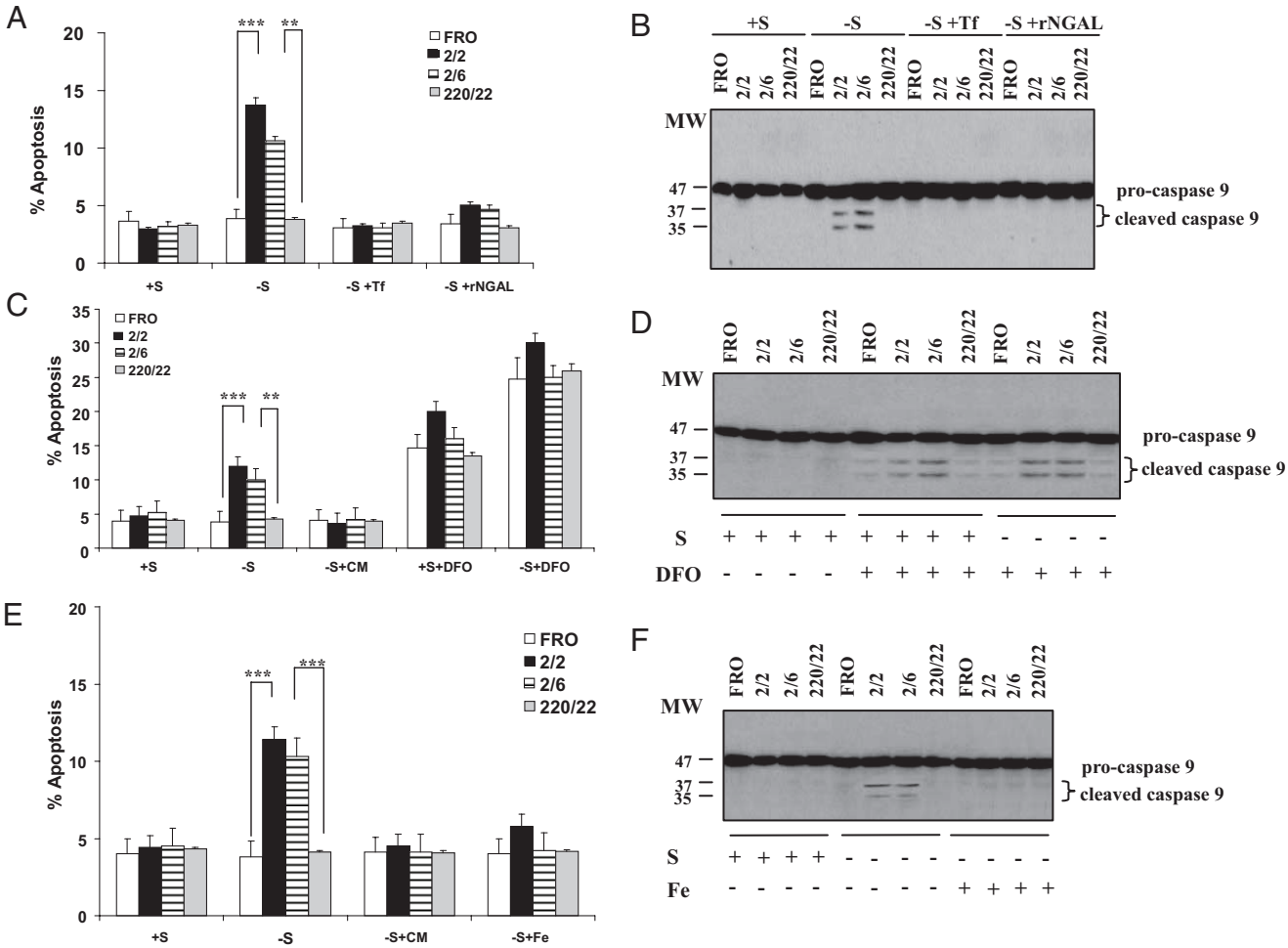

Fig. 4. Iron mimics the role of NGAL-mediated survival of FRO cells. 2.5×10^5 cells per well were seeded in 6-well culture plates and grown in different experimental conditions, as indicated. Cell death was either assessed by propidium iodide staining (*A*, *C*, and *E*) or by Western blot analysis (*B*, *D*, and *F*). Results were mean ± SD of at least three separate experiments. S, serum; CM, conditioned medium; Tf, transferrin; rNGAL, recombinant NGAL; DFO, deferoxamine; Fe, iron. **, $P < 0.0005$; ***, $P < 0.0001$.

FRO cells, FRO Neo cells, or 220/22 cells, to 2/2 and 2/6 clones. The same effect was also seen in the FRO cells transiently transfected with IκBαM (Fig. S3*D*). To confirm the activation of the apoptotic machinery, we analyzed by using Western blot analysis, the caspase-9 activity in parental FRO cells and NGAL siRNA clones grown in the same experimental conditions used in the propidium iodide assay (Fig. 3*B*). Serum deprivation induced cleavage of caspase-9 in 2/2 and 2/6 clones but not in parental FRO cells (Fig. 3*B*). Also, in this case, incubation of NGAL siRNA clones with the conditioned medium of FRO cells, FRO Neo cells, or 220/22 cells restored their survival (Fig. 3 *A* and *B*). These data strongly suggest that NGAL is a survival factor for thyroid carcinoma cells.

A dissection of the apoptosis pathways is illustrated in Fig. S4. In the NGAL siRNA clones grown in absence of serum, the initiating caspases-2 and -9 and the execution caspases-3 and 7 are activated, whereas the activity of other caspases (such as the initiating-, receptor-associated caspase-8) are not. This effect is reverted by the addition of either recombinant NGAL or iron (Fig. S4*A*). A very similar pattern of caspases activation is detected in FRO or 220/22 cells incubated with deferoxamine (DFO). Iron deprivation also resulted in the decrease of mitochondrial membrane potential (Fig. S4*B*) and in the release of cytochrome C (Fig. S4*C*). These data suggest that iron deprivation induces apoptosis via collapse of the mitochondrial membrane potential, release of cytochrome C from mitochondria, and activation of execution caspases.

NGAL-Mediated Intake of Extracellular Iron Accounts for the Antiapoptotic Activity of NGAL in FRO Cells. One of the properties of NGAL is an ability to deliver iron from the extracellular milieu into the cells. Because it has been described that iron could play an important role in cancer and apoptosis (22), we tested whether the increased susceptibility of siRNA NGAL clones to cell death was because of the decreased NGAL-mediated delivery of iron. We first analyzed the ability of iron-loaded transferrin and NGAL to rescue siRNA NGAL clones from serum deprivation-induced apoptosis. As reported in Fig. 4, both propidium iodide staining (*A*) and Western blot anti-caspase-9 (*B*) showed that addition of transferrin or recombinant NGAL to the serum-free medium restored the survival of siRNA clones.

To further confirm that NGAL acts as a survival factor for cancer cells by delivering iron, we used DFO (an iron chelator) and ferric chloride ($FeCl_3$, an iron source) to evaluate the survival of the siRNA clones. When cells were grown in complete medium in presence of DFO, both parental FRO cells and derived siRNA clones underwent apoptosis (Fig. 4 *C* and *D*). However, $FeCl_3$ produced the opposite effect; it restored the resistance of siRNA NGAL clones to serum deprivation-induced apoptosis (Fig. 4 *E* and *F*).

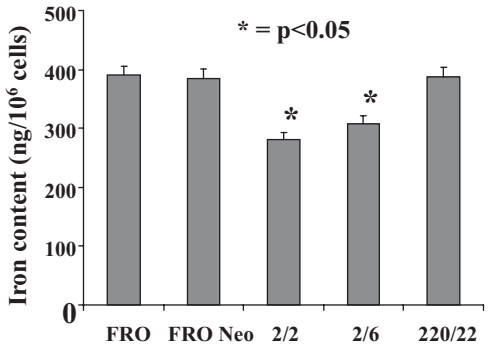

Fig. 5. Intracellular iron content of the different FRO cell lines. Colorimetric analysis of intracellular iron concentration is shown. Results were mean ± SD of at least three separate experiments, performed in triplicate. Significantly different from controls. *, $P < 0.05$.

Because these results indicated that the intracellular iron content could be relevant for the survival of FRO cells, we analyzed the intracellular iron concentration of parental FRO cells and NGAL siRNA clones (Fig. 5). The colorimetric assay showed that 2/2 and 2/6 clones lacked at least 20–30% of iron content ($P < 0.05$) compared with FRO, FRO Neo, and 220/22 cells, thus suggesting that the absence of NGAL determined a decrease of iron uptake in the clones.

Discussion

We have identified NGAL as a mediator of the NF-κB oncogenic activity in thyroid cancer. NGAL is highly expressed in the human thyroid carcinoma FRO cell line and other poorly differentiated thyroid cancer cell lines (Fig. 1*A* and data not shown), is highly expressed in primary human ATC (Fig. 1 *B–E* and Table 1), and acts as a survival factor for thyroid cancer cells (Figs. 3 and 4). The prosurvival activity of NGAL is mediated by its ability to bind iron and to transport it inside the cells (Figs. 4 and 5). These findings show that NGAL is a critical effector of NF-κB-mediated oncogenic activity, defines a prosurvival function of NGAL, and highlights iron as a central controller of cell survival.

Various types of cancers express high levels of NGAL, including colon, pancreas, breast, bladder, and liver (9–13). NGAL represents the human homolog of the rat neu-related lipocalin, a gene that was shown to be overexpressed in HER-2/neu oncogene-induced rat mammary tumors (23). Whether NGAL is causal or contributory to cancer is unknown. It has been reported that NGAL is a surviving factor for cancer cells. Ectopic expression of NGAL in lung and breast cancer cell lines reduced the apoptosis that was induced by a PDK1 inhibitor whereas the decreased expression of NGAL by siRNA had opposite effects (18). Our results confirm that NGAL is a surviving factor for cancer cells, and we extend these findings by demonstrating that the protective effect of NGAL is mediated by its ability to bind and transport iron. Our results are in agreement with the model proposed by Devireddy *et al.* (17). In this model, it is proposed that internalization of the apo form of NGAL leads to iron loss and apoptosis. Conversely, internalization of iron-loaded NGAL might prevent apoptosis.

Iron contributes to enzyme activity in DNA synthesis, metabolism, and oxygen response, and its acquisition plays a critical role in development, cell growth, and survival (22). Cancer cells have a higher requirement for iron than normal cells because they rapidly proliferate. This is reflected by the evidence that tumor cells have higher numbers of transferrin receptors on their surface, mediating a high rate of iron uptake (24). The importance of keeping the levels of iron uptake constant is further suggested by the evidence that multiple and redundant systems for iron uptake and transport exist. Most cells acquire iron by capturing iron-loaded transferrin. However, hypotransferrinemic mice (25, 26) and humans (27) have defects in central nervous system development and hematopoiesis when most epithelial organs are normal. Likewise, the mice lacking the transferrin receptor 1 initiate organogenesis but succumb to the effects of anemia (28). Given that iron is necessary for all of the cells, there must be other pathways for iron acquisition in epithelial cells. One such pathway is mediated by NGAL. In our experimental model, we observed that cells knocked down for NGAL expression showed a decrease of ≈20–30% iron content, and that this decrease still allowed cell survival. However, whether either the iron delivery was further decreased or cells were exposed to an appropriate stress, such as serum deprivation, cells underwent apoptosis. Indeed, administration of iron, either as iron salt, transferrin, or iron-loaded NGAL, restored the ability of knockdown cells to survive in the absence of serum. Similarly, NGAL knockout mice are vital and do not show gross developmental defects, although they succumb to bacterial infection (29).

Many studies have demonstrated that iron chelators, such as DFO, have effective anticancer activity (30). Iron is essential for the catalytic activity of ribonucleotide reductase (an enzyme mediating the conversion of all four ribonucleotides to their deoxyribonucleotide counterparts), which is the rate-limiting step of DNA synthesis (31). However, in our experimental system, the proliferation rate of the cells knocked down for NGAL expression is not affected (data not shown).

Iron levels strictly control the activity of specific prolyl hydroxylase-domain enzymes (PHDs), which, in turn, promote functional activation of transcription factors involved in tumor development, as is the case of the HIF-1, which controls genes involved in energy metabolism and angiogenesis (32). HIF-1 is primarily regulated by specific PHDs that initiate its degradation via the von Hippel-Lindau tumor suppressor protein. The oxygen and iron dependency of PHD activity accounts for regulation of the pathway by both cellular oxygen and iron status. We have evidence that in our experimental system, the protein level of HIF-1α is increased in the NGAL knockdown clones and is down-regulated by the addition of iron. Conversely, in control cells, the level of HIF-1α protein is low and up-regulated by the addition of DFO. Moreover, the activity of a HIF1-responsive promoter parallels the level of HIF-1 protein (A.I., F.P., and A. Leonardi, unpublished observation). Because NF-κB is a central component in the hypoxic response that positively regulates HIF-1α expression (33), it is tempting to speculate that the involvement of NGAL as an iron transporter, and its opposing effect on HIF-1α expression, could be a part of an autoregulatory loop that has inhibitory function between NF-κB and HIF-1 to control tumor progression.

Modulation of cell survival may not be the only way NGAL influences the behavior of a cancer cell. NGAL has been demonstrated to form a complex with MMP-9, playing a role in the maintenance of an extracellular pool of a potentially active form of the protease, whose activity is associated with angiogenesis and tumor growth (16). This is supported by the evidence that the level of NGAL expression correlates with the clinical outcome of the patients and the depth of the tumor invasion (17). We have evidence that a complex between NGAL and MMP-9 also exists in transformed thyroid cells, although neither the exact role that such a complex plays in thyroid cancer, nor whether or not the activity of MMP-9 is affected by the presence of NGAL, is known (A.I. and A. Leonardi, unpublished observations).

In other experimental systems, NGAL has been shown to induce expression of E-cadherin to promote formation of polarized epithelia and diminish invasiveness of ras-transformed

cell lines (34). In our experimental system, NGAL seems to have a different role. In fact, ectopic expression of NGAL in a normal thyroid cell line, or its knockdown in transformed cells, does not alter the expression of E-cadherin or vimentin (data not shown).

Our results, in addition to identifying NGAL as a potential target for therapeutic intervention, also strengthen the rationale for the use of iron chelators in the treatment of cancer. Depleting iron from a rapidly dividing cancer cell through the implementation of iron chelators, or decreasing NGAL expression, deprives iron of a component critical for various cellular processes and induces apoptosis.

Materials and Methods

Cell cultures and biological reagents, processing of conditioned media, two-dimensional gel elecrophoresis, mass spectrometry analysis, immunohistochemical analysis, Northern and Western blot experiments, *in vitro* and *in vivo* tumorigenicity assays, measurements of apoptosis, quantification of iron content, and statistical analysis were performed as described in the *SI Text*.

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