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Negative feedback regulation of NF-κB-inducing kinase is proteasome-dependent but does not require cellular inhibitors of apoptosis

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ABSTRACT

Non-canonical NF-κB signaling is controlled by the precise regulation of NF-κB inducing kinase (NIK) stability. NIK is constitutively ubiquitylated by cellular inhibitor of apoptosis (cIAP) proteins 1 and 2, leading to its complete proteasomal degradation in resting cells. Following stimulation, cIAP-mediated ubiquitylation of NIK ceases and NIK is stabilized, allowing for inhibitor of κB kinase (IKK)α activation and non-canonical NF-κB signaling. Non-canonical NF-κB signaling is terminated by feedback phosphorylation of NIK by IKKα that promotes NIK degradation; however, the mechanism of active NIK protein turnover remains unknown. To address this question, we established a strategy to precisely distinguish between basal degradation of newly synthesized endogenous NIK and induced active NIK in stimulated cells. Using this approach, we found that IKKα-mediated degradation of signal-induced activated NIK occurs through the proteasome. To determine whether cIAP1 or cIAP2 play a role in active NIK turnover, we utilized a Smac mimetic (GT13072), which promotes degradation of these E3 ubiquitin ligases. As expected, GT13072 stabilized NIK in resting cells. However, loss of the cIAPs did not inhibit proteasome-dependent turnover of signal-induced NIK showing that unlike the basal regulatory mechanism, active NIK turnover is independent of cIAP1 and cIAP2. Our results therefore establish that the negative feedback control of IKKα-mediated NIK turnover occurs via a novel proteasome-dependent and cIAP-independent mechanism.

1. Introduction

NF-κB activation occurs by two separate mechanisms: the classical and non-canonical NF-κB pathways. Classical NF-κB signaling is vital for host immunity, cellular survival and proliferation, and the inflammatory response [1,2]. Non-canonical NF-κB activity is required for lymph node organogenesis and B cell development [3–5]; however, increasing evidence implicates aberrant non-canonical signaling in chronic inflammatory diseases and cancers [5–7]. Consequently, defining the mechanisms that regulate both the activation and down-regulation of non-canonical NF-κB signaling is crucial for the development of novel therapeutic strategies to selectively block this pathway.

Non-canonical NF-κB signaling is controlled by NF-κB inducing kinase (NIK) protein stability [3,5]. NIK is continuously translated in resting cells [8] but is normally undetectable due to the activity of a regulatory complex containing TRAF2, TRAF3, cIAP1 and cIAP2. NIK associates with this complex by binding TRAF3, which associates with cIAP1/2-bound TRAF2 [9–11]. cIAP1 and cIAP2 are E3 ubiquitin ligases which target newly synthesized NIK for proteasomal degradation in unstimulated cells [11,12]. The cIAPs act redundantly, as silencing both cIAP1 (*Birc2*) and cIAP2 (*Birc3*) is required to stabilize NIK in resting cells [11]. Following ligation of receptors such as the lymphotoxin-β receptor (LTβR) that activate the non-canonical pathway, the ubiquitin ligase activity of cIAP1/2 is redirected to TRAF3, which releases NIK from the negative regulatory complex and permits its stabilization [13].

Abbreviations: NIK, NF-κB inducing kinase; cIAP, cellular inhibitors of apoptosis; IKK, inhibitor of κB kinase.

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Stable NIK is constitutively active [14] and phosphorylates and activates inhibitor of kappa-B kinase (IKK)α. Active IKKα phosphorylates the NF-κB precursor p100 leading to its proteasomal processing to p52. p52 bound to RelB translocates to the nucleus to regulate a discrete panel of genes involved in lymph node organogenesis and B cell maturation [15,16].

The duration of non-canonical NF-κB signaling is controlled by a crucial negative regulatory mechanism in which activated IKKα feeds back to phosphorylate NIK at C-terminal serine residues [17]. These modifications promote NIK turnover, however, the precise mechanism of IKKα-induced NIK degradation remains unknown. Overexpression and inhibitor studies suggest that the TRAF2/3:cIAP1/2 complex that controls the basal turnover of newly synthesized NIK does not require prior NIK phosphorylation [17]. Importantly, these experiments did not directly address a potentially distinct role for the cIAPs in controlling the turnover of activated endogenous NIK. As the cIAPs are, to date, the only known regulators of basal NIK stability, we sought to determine whether cIAP1 and cIAP2 are required for the negative turnover of active NIK.

To precisely differentiate between the endogenous degradation of newly synthesized NIK in resting cells and active NIK in LTβR-stimulated cells, we employed proteasome and protein synthesis inhibitors. Using this approach we have definitively determined that signal-induced active NIK is rapidly degraded by the proteasome. Furthermore, we used a novel Smac mimetic that promotes degradation of cIAP1 and cIAP2, and found that unlike the basal regulatory mechanism, active endogenous NIK turnover does not require the cIAPs. Our findings establish that the negative feedback control of IKKα-induced NIK turnover occurs via a novel proteasome-dependent but cIAP-independent mechanism.

2. Materials and methods

2.1. Cell lines and culture

3T3-NIH Mouse Embryonic Fibroblasts and HeLa cells were from ATCC. IKKα^{KO} MEFs were from Inder Verma (The Salk Institute, San Diego, CA). All cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologics), 2 mM L-glutamine, penicillin (50 units/mL) and streptomycin (50 units/mL). Cells were stimulated when they reached 80% confluence.

2.2. Antibodies and reagents

Recombinant human LIGHT, anti-cIAP1, and pan-cIAP antibodies were from R&D Systems. Anti-NIK and -mouse p100/p52 were from Cell Signaling Technology. Anti-human p100/p52 was from Millipore. Anti-IκBα was from Santa Cruz Biotechnology and anti-NEMO was from MBL International. Anti-IKKα was from Imgenex and monoclonal anti-cIAP1 was from Enzo Life Sciences. Horseradish Peroxidase-conjugated secondary antibodies were from Jackson Immuno Research. Agonistic antibody against the LTβR (5G11) was from Abcam. Protein λ-phosphatase was from New England Biosciences. Anti-α-Tubulin and MG132 were from Sigma–Aldrich. Bortezomib was a generous gift from Katherine A. High, M.D. (The Children's Hospital of Philadelphia). Bortezomib was used at a final concentration of 500 nM. Cells were treated with 0.05% DMSO as a control. Cycloheximide was from EMD Millipore and used a final concentration of 2.5 μg/mL. As a control, cells were treated with 0.1% ethanol. The active Smac mimetic GT13072 and the inactive Smac mimetic GT13199 were generously provided by TetraLogic Pharmaceuticals (Malvern, PA). Smac mimetics were used at a final concentration of 1 μM. Cells were treated with 0.1% DMSO as a control.

2.3. Immunoblotting

Cells were harvested on ice in a lysis buffer consisting of 150 mM NaCl, 50 mM TricCl pH 7.5, 1% Triton X-100, and complete protease inhibitors (Roche). Twenty micrograms of total protein was separated by SDS–PAGE and transferred onto PVDF membrane (Millipore). The immunoblots presented are one of three representative experiments developed using enhanced chemiluminescence.

3. Results

3.1. Active NIK is turned over rapidly by the proteasome

To examine NIK stabilization in WT MEFS, we incubated cells with LIGHT (TNFSF14) for up to eight hours. As expected, NIK was in low abundance in resting WT cells but was detected following treatment with LIGHT, with maximal levels occurring after four hours of incubation (Fig. 1A). Consistent with a negative regulatory role for IKKα in controlling NIK turnover [17], abundant NIK was detected in resting IKKα^{KO} MEFs and could be further elevated with LIGHT stimulation (Fig. 1A). Notably, the electrophoretic mobility of NIK in both resting and LIGHT-stimulated IKKα^{KO} MEFs was greater than active NIK in WT MEFs, which was consistently detected as a doublet (Fig. 1A, compare lanes 5 and 11). We incubated samples with λ-phosphatase to determine that active NIK in WT MEFs was phosphorylated (Fig. 1B, lanes 1 and 2). In contrast, the lower molecular weight NIK in IKKα^{KO} cells was unaffected by phosphatase treatment indicating that NIK is unphosphorylated in the absence of IKKα (Fig. 1B).

To specifically analyze the turnover of active NIK, MEFs were stimulated with LIGHT to increase NIK abundance then incubated with cycloheximide (CHX) to block protein synthesis. This approach allowed us to exclude newly synthesized NIK and specifically examine the turnover of LIGHT-activated NIK. As shown in Fig. 1C (left panel), active NIK in WT MEFs was rapidly turned over during CHX treatment and was undetectable within 45 min of CHX chase. In contrast, LIGHT-induced NIK in IKKα-deficient MEFs remained elevated above basal amounts even after sixty minutes of CHX (Fig. 1C; right panel) confirming that IKKα is required for efficient turnover of active NIK. Together these findings support the IKKα-dependent negative feedback model for non-canonical NF-κB signaling termination [17]. Importantly, these data also establish that active NIK turnover is rapid, with complete loss of detectable NIK occurring within 30–45 min of signal-induced release from the basal regulatory complex.

We next asked if the proteasome plays a role in the IKKα-dependent turnover of endogenous signal-induced NIK. Consistent with previous reports [8–11,13,18], pharmacological blockade of the proteasome using MG132 led to NIK accumulation in resting cells (Fig. 2A, lane 2), indicating that basal NIK is constitutively degraded through the proteasome. However, analysis of proteasome inhibition on the fate of signal-induced NIK is complicated by concomitant effects on basal NIK levels (Fig. 2A). We therefore used a combination of proteasome and protein synthesis inhibition to isolate signal-induced NIK. Importantly, NIK was undetectable in resting cells co-treated with MG132 and CHX, confirming that in the absence of proteasome function, protein synthesis inhibition prevents NIK accumulation (Fig. 2A). We therefore employed this strategy to determine the effects of proteasome inhibition on active NIK turnover. As shown in Fig. 2B, LIGHT treatment induced NIK accumulation in WT MEFs and further incubation in CHX led to the loss of detectable NIK (lane 3). Moreover, inhibition of the proteasome with concomitant CHX treatment inhibited LIGHT-induced NIK turnover (lane 4), indicating that NIK degradation is proteasome-dependent. Notably, inhibition of the proteasome in

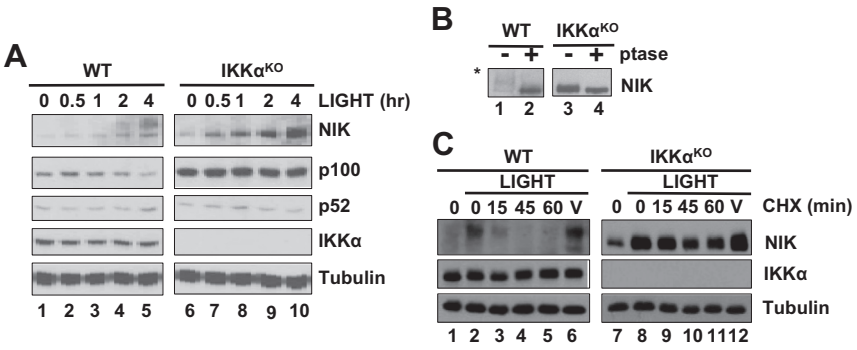

Fig. 1. IKKα is required for the turnover of active NIK. (A) WT or IKKα-deficient (IKKα^{KO}) MEFs were treated with LIGHT (100 ng/ml) for the times indicated. Whole cell extracts were immunoblotted for the indicated proteins. (B) WT or IKKα^{KO} MEFs were treated with LIGHT for four hours to stabilize NIK. Whole cell extracts were then treated with protein λ-phosphatase and immunoblotted for NIK. * Indicates the phosphorylated protein. (C) WT or IKKα^{KO} MEFs were treated as in (B), followed by 2.5 μg/ml cycloheximide (CHX) or vehicle (V) for the times indicated. Lysates were immunoblotted for the indicated proteins.

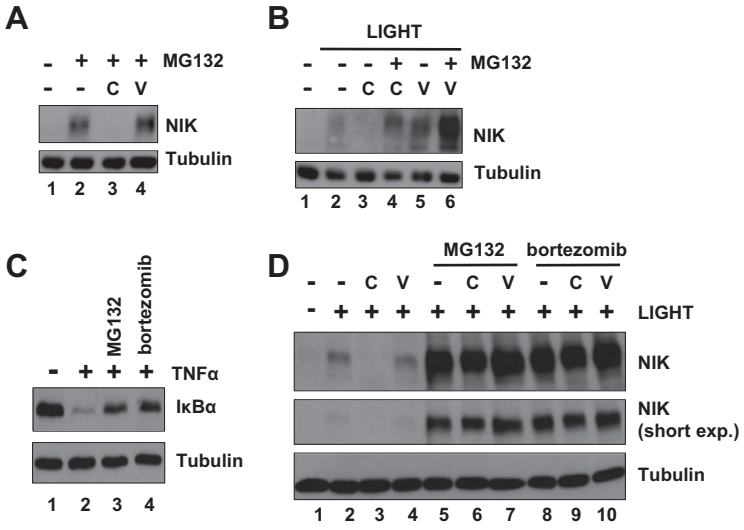

Fig. 2. Active NIK is degraded via the proteasome in MEFs. (A) WT MEFs were treated with 10 μM MG132 (+), or MG132 plus 2.5 μg/ml cycloheximide (C) or vehicle control (V) for one hour. Whole cell lysates were immunoblotted with antibodies against the indicated proteins. (B) WT MEFs were treated with LIGHT for four hours followed by one hour of cycloheximide (C) or vehicle (V) in the absence or presence of MG132 (+) to inhibit the proteasome. Lysates were immunoblotted as in (A). (C) WT MEFs were either left untreated or incubated with 500 nM MG132 or 500 nM bortezomib for 8 h. Cells were then stimulated with TNF for 15 min. Lysates were immunoblotted with antibodies against the indicated proteins. (D) WT MEFs were treated with 500 nM MG132 or 500 nM bortezomib, with (+) or without (−) LIGHT for 6.5 h and subsequently incubated with cycloheximide (C) or vehicle (V) for 90 min. Lysates were immunblotted as in (A).

the absence of CHX led to increased accumulation of NIK in LIGHT-stimulated cells (Fig. 2B, lane 6), further signifying that CHX treatment isolated active NIK from the constitutively translated basal protein.

As MG132 may have off-target effects *in vitro* [19], we also utilized the more selective inhibitor bortezomib (Velcade, PS-341) to confirm the role of the proteasome in active NIK turnover. Similar to MG132, bortezomib inhibits the chymotrypsin-like enzymatic site of the 20S core particle [20]. As expected, both inhibitors blocked TNF-induced proteasome-dependent degradation of IκBα in the classical NF-κB signaling pathway [1] (Fig. 2C). Importantly, the turnover of LIGHT-induced NIK was blocked by pretreatment with either MG132 or bortezomib (Fig. 2D). These data establish, with two different pharmacological inhibitors, that the IKKα-dependent turnover of LIGHT-induced endogenous NIK is proteasome-dependent.

3.2. Turnover of active NIK is independent of cIAP1 and cIAP2

The sensitivity of NIK turnover to proteasome inhibition suggests that active NIK is ubiquitylated, thereby targeting it for degradation. As cIAP1 and cIAP2 (cIAP1/2) are currently the only E3 ubiquitin ligases implicated in NIK regulation [10,11], we hypothesized that in addition to controlling the degradation of newly synthesized NIK, cIAP1/2 also ubiquitylated active, phosphorylated NIK. To assess the requirement of cIAP1/2 in NIK turnover, we used a Smac mimetic compound (GT13072) to degrade endogenous cIAP1/2 [21]. GT13072 treatment led to the rapid loss of cIAP1 and induction of NIK in resting MEFs, whereas an inactive compound (GT13199) did not (Fig. 3A). GT13072 treatment induced NIK stabilization and p100 processing more rapidly than LIGHT (Fig. 3B). Furthermore, GT13072 treatment induced a second NIK band in WT MEFs but not IKKα^{KO} MEFs (Fig. 3C), and λ-phosphatase treatment showed that this band represents phosphorylated NIK (Fig. 3D). Thus release of NIK from the control of the TRAF2/3:cIAP1/2 complex promotes its stabilization and phosphorylation similar to that induced by the natural ligand LIGHT or a LTβR cross-linking antibody (Fig. 3C, lane 11). We therefore used GT13072 to determine if cIAPs are further required to promote turnover of active NIK. To specifically isolate GT13072-induced NIK from basally translated NIK, we employed the CHX chase approach established in Fig. 1C. Similar to LIGHT-induced NIK, NIK stabilized

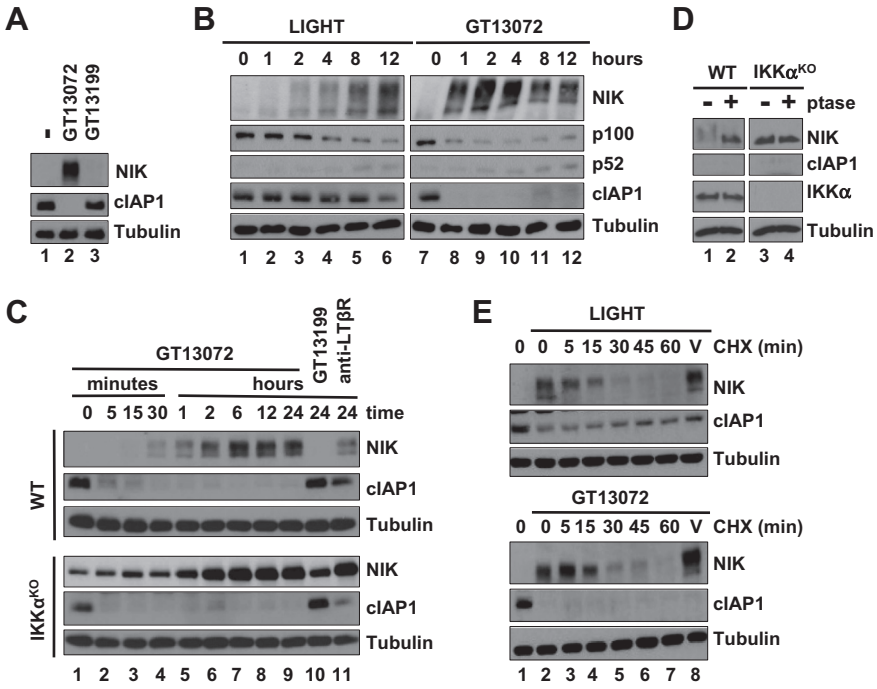

Fig. 3. The Smac mimetic GT13072 does not block NIK turnover in MEFs. (A) WT MEFs were treated for four hours with either 1 μM GT13072 or the inactive compound GT13199. Cells were lysed and immunoblotted for the indicated proteins. (B) WT MEFs were treated with LIGHT or GT13072 for the times indicated. Lysates were immunoblotted for the indicated proteins. (C) WT or IKKα^{KO} MEFs were treated with GT13072, GT13199, or anti-LTβR Ab for up to 24 h. Lysates were immunblotted as in (A). (D) WT or IKKα^{KO} MEFs were treated with GT13072 for four hours to stabilize NIK. Whole cell extracts were treated with protein λ-phosphatase and immunoblotted for NIK. (E) WT MEFs were stimulated with either LIGHT or GT13072 for four hours, followed by either 2.5 μg/ml cycloheximide (CHX) for up to one hour or vehicle (V) for one hour. Lysates were immunoblotted as in (A).

by GT13072 was turned over within sixty minutes of CHX treatment, despite the absence of cIAP1 (Fig. 3E). Thus, cIAP1 is dispensable for the turnover of active NIK in MEFs.

Although we were unable to detect resting cIAP2 protein in MEFs, NIK was stabilized by GT13072 treatment alone (Fig. 3A). As cIAP1 and cIAP2 have been shown to be functionally redundant in basal NIK regulation [11], this suggested that GT13072 also degraded any endogenous cIAP2. However, to definitively determine that cIAP1 and cIAP2 do not play distinct roles in active NIK turnover, we assessed NIK regulation in HeLa cells, which express both cIAP1 and cIAP2 (Fig. 4A). GT13072 treatment led to the rapid loss of cIAP1 and cIAP2 in HeLa cells followed by NIK stabilization (Fig. 4A). As in MEFs, treatment with GT13072 induced p100 processing similar to the natural ligand LIGHT (Fig. 4B). Importantly, using CHX to distinguish active NIK from basally translated protein revealed that negative regulation of active NIK was intact in HeLa cells after cIAP1/2 degradation (Fig. 4C). These results thereby establish that endogenous cIAP1 and cIAP2 are dispensable for the turnover of active NIK.

3.3. NIK turnover in the absence of cIAP1/2 occurs through the proteasome

Thus far we have established that active NIK turnover induced by LIGHT requires the proteasome. Moreover, turnover of GT13072-stabilized NIK occurs in the absence of cIAP1/2 in both MEFs and HeLa cells. We therefore questioned whether GT13072-induced NIK turnover required the proteasome. To specifically follow the destabilization of active NIK induced by GT13072, we employed the co-treatment strategy described in Fig. 2. Similar to the effects in MEFs (Fig. 2A), treatment of HeLa cells with MG132 stabilized basal NIK, a process that was dependent on new protein synthesis (not shown). We next induced NIK using GT13072 and assessed the effect of MG132 on NIK turnover. Fig. 4 shows that in both HeLa cells and MEFs, NIK turnover is blocked by co-incubation of MG132 with CHX (Fig. 4D, lanes 3 and 4). Thus we conclude that unlike basal NIK turnover, which absolutely requires cIAP1/2 [10,11], proteasome-dependent turnover of active endogenous NIK does not require the cIAPs. Together, our data support a model in which the negative feedback control of IKKα-induced NIK turnover is proteasome-dependent but cIAP-independent (Fig. 4E).

4. Discussion

Pathologies associated with aberrant non-canonical NF-κB signaling underscore the importance of fully elucidating the mechanisms of NIK regulation. Genetic alterations in the basal regulatory complex have been associated with multiple myeloma and melanoma in humans. [7,22–24]. More recently, blocking either the ubiquitin ligase activity of cIAP2 or the negative turnover of active NIK have been shown to result in B cell hyperplasia and kidney nephropathy in mice [25,26]. As controlling non-canonical NF-κB signaling depends on the stabilization of NIK, defining NIK regulatory mechanisms is crucial for the development of novel therapeutic strategies aimed at selectively manipulating this pathway.

IKKα-mediated negative feedback control of non-canonical NF-κB signaling requires the phosphorylation of NIK [17]. Here we show that this post-translational modification directs the active kinase to the proteasome for degradation. The most common mechanism of signal-induced proteasomal targeting is ubiquitylation [27,28], and previous overexpression studies have shown that NIK can be ubiquitylated by exogenous cIAP1 or cIAP2 [10,11,17]. Yet these experiments did not distinguish between ubiquitylation of basally translated NIK and NIK stabilized by activating ligands.

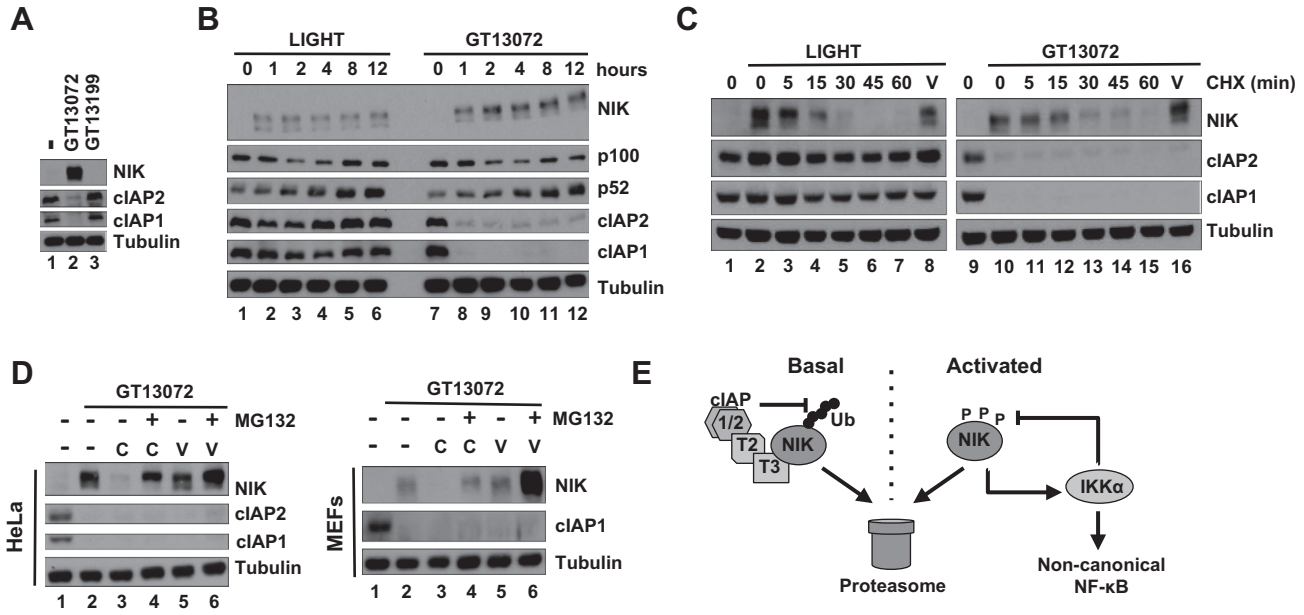

Fig. 4. cIAP1/2 are not required for proteasome-dependent NIK turnover in HeLa cells. (A) HeLa cells were treated for four hours with either 1 μM GT13072 or GT13199. Cells were lysed and immunoblotted for the indicated proteins. (B) HeLa cells were treated with LIGHT or GT13072 for the times indicated. Lysates were immunoblotted for the indicated proteins. (C) HeLa cells were stimulated with either LIGHT or GT13072 for four hours. Cells were then treated with 2.5 μg/ml cycloheximide (CHX) for up to one hour (V, vehicle control). Cells were lysed and immunoblotted for the indicated proteins. (D) HeLa cells or WT MEFs were treated as in (C) plus 10 μM MG132 (+) for one hour (C, cycloheximide; V, vehicle control). Lysates were immunoblotted for the indicated proteins. (E) In unstimulated cells scant NIK abundance is maintained by a complex consisting of TRAF2 (T2), TRAF3 (T3) and cIAP1/2. The cIAPs ubiquitylate newly synthesized NIK (black circles) leading to its rapid proteasomal degradation (*left side*). Following ligation of receptors that activate non-canonical NF-κB, the basal regulatory complex is disrupted and NIK protein increases to detectable levels. Accumulated NIK then associates with and activates IKKα leading to induction of non-canonical NF-κB (*right side*). Activated IKKα also feeds back to phosphorylate NIK (P) triggering its degradation by the proteasome. Unlike basal NIK turnover, this feedback degradation of active NIK does not require cIAP1/2.

We have previously shown that TRAF3 is dispensable for active NIK turnover [29]. However, since cIAP1/2 are the only E3 ubiquitin ligases known to target NIK directly, we hypothesized that cIAP1/2 might play a TRAF3-independent role in the turnover of active NIK. Our results using the Smac mimetic GT13072 indicate that loss of cIAP1/2 has no effect on the turnover of active NIK. Thus we conclude that ubiquitylation of NIK by cIAP1/2 is limited to only the newly synthesized protein controlled by the basal TRAF3:TRAF2 regulatory complex. Our data therefore suggest that phosphorylated NIK is ubiquitylated by a currently unidentified E3 ubiquitin ligase. One potential candidate is $SCF^{\beta TrCP}$, which ubiquitylates IκBα in the classical NF-κB pathway [1]. Whether βTrCP or another E3 ubiquitin ligase controls active NIK turnover will be an important area of future research to selectively manipulate the duration of non-canonical NF-κB activity.

Our finding that the turnover of NIK absolutely requires the proteasome strongly suggests that active NIK is ubiquitylated. Despite intense efforts, we have been unable to detect ubiquitylation of endogenous NIK under any conditions. As we have ruled out the cIAPs as E3 ubiquitin ligases targeting NIK, an alternative possibility is that active NIK is phosphorylated by IKKα and degraded by the 20S proteasome via a ubiquitin-independent mechanism. While such a mechanism is rare, several proteins including p53 and ornithine decarboxylase (ODC) can be degraded "by default" [28,30,31]. Our results cannot conclusively eliminate this possibility and ubiquitin-independent degradation of active NIK is an intriguing mechanism to consider.

Collectively our findings separate the molecular requirements for active NIK turnover from those involved in the basal regulation of newly translated protein, revealing a previously unappreciated layer of NIK regulation. Increasing reports of dysregulated non-canonical NF-κB signaling in disease [4,6,7] and the critical role of NIK stabilization in non-canonical NF-κB [3] underscore the importance of fully elucidating the mechanism of active NIK turnover to develop novel therapeutic strategies to terminate the non-canonical NF-κB signaling pathway.

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