

# Recruitment of A20 by the C-terminal domain of NEMO suppresses NF- $\kappa$ B activation and autoinflammatory disease

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**Receptor-induced NF- $\kappa$ B activation is controlled by NEMO, the NF- $\kappa$ B essential modulator. Hypomorphic NEMO mutations result in X-linked ectodermal dysplasia with anhidrosis and immunodeficiency, also referred to as NEMO syndrome. Here we describe a distinct group of patients with NEMO C-terminal deletion ( $\Delta$ CT-NEMO) mutations. Individuals harboring these mutations develop inflammatory skin and intestinal disease in addition to ectodermal dysplasia with anhidrosis and immunodeficiency. Both primary cells from these patients, as well as reconstituted cell lines with this deletion, exhibited increased I $\kappa$ B kinase (IKK) activity and production of proinflammatory cytokines. Unlike previously described loss-of-function mutations,  $\Delta$ CT-NEMO mutants promoted increased NF- $\kappa$ B activation in response to TNF and Toll-like receptor stimulation. Investigation of the underlying mechanisms revealed impaired interactions with A20, a negative regulator of NF- $\kappa$ B activation, leading to prolonged accumulation of K63-ubiquitinated RIP within the TNFR1 signaling complex. Recruitment of A20 to the C-terminal domain of NEMO represents a novel mechanism limiting NF- $\kappa$ B activation by NEMO, and its absence results in autoinflammatory disease.**

NF- $\kappa$ B | autoinflammatory disease | A20 | HED-ID | TLR

Activation of the NF- $\kappa$ B family of transcription factors is required for normal development, innate and adaptive immunity, and the inflammatory response (1, 2). NF- $\kappa$ B-induced transcription of proinflammatory cytokines and chemokines amplifies immune-response programs and stimulates recruitment of inflammatory cells. Transcriptional activity of the classic NF- $\kappa$ B p65/p50 complex is regulated by the inhibitor of NF- $\kappa$ B kinase (IKK), consisting of the  $\alpha$ - and  $\beta$ -catalytic subunits and the NEMO (NF- $\kappa$ B essential modulator, IKK $\gamma$ ) regulatory subunit. IKK activity leads to phosphorylation and K48-linked polyubiquitination of I $\kappa$ B $\alpha$ , the inhibitor of NF- $\kappa$ B. Ubiquitinated I $\kappa$ B $\alpha$  is then rapidly degraded, allowing nuclear translocation of NF- $\kappa$ B subunits and gene transactivation. NEMO functions as a scaffold within the IKK complex that is required for canonical IKK enzymatic activity and NF- $\kappa$ B activation (3).

Activation of TNF, IL-1R, and Toll-like receptor (TLR) family receptors leads to K63 and linear (also termed M1) ubiquitination of cytosolic adapter proteins, such as receptor interacting protein 1 (RIP1). This in turn promotes recruitment of the IKK complex to the receptor signaling complex (4–8). A well-known negative regulator of NF- $\kappa$ B activation is A20 (encoded by the gene *TNFAIP3*). In the TNF-R1 complex, A20 removes K63-linked ubiquitin modifications on RIP1 through its deubiquitinase activity and converts these to K48-linked polyubiquitin chains through its E3 ligase activity (9). Editing of

polyubiquitin linkages at the receptor complex in this manner results in rapid degradation of RIP1 and other signaling proteins, such as TRAF6, TRAF2, cIAP1, and cIAP2 (9, 10), promoting the termination of receptor-induced NF- $\kappa$ B activation. In addition, A20 directly inhibits IKK activity independently of its deubiquitinase activity in a manner that depends upon binding to NEMO (11). A20-deficient cells demonstrate prolonged NF- $\kappa$ B activation and elevated production of inflammatory cytokines in response to TNF and TLR ligands. A20-deficient mice are hypersensitive to TNF and LPS-induced septic shock and spontaneously develop multiorgan inflammation and cachexia that results in premature mortality (12, 13).

The importance of regulated NF- $\kappa$ B activation in humans is illustrated by the effect of hypomorphic NEMO mutations, which result in a combination of immune and developmental defects. Ectodermal dysplasia with anhidrosis and immunodeficiency (EDA-ID) is a pleomorphic X-linked disorder affecting the development of ectodermally derived structures (eccrine glands, hair follicles, and teeth), as well as innate and adaptive immunity (14). Immunodeficiency, marked by the frequency and severity of

## Significance

Regulated activation of the NF- $\kappa$ B family of transcription factors is important for normal development, immune cell function, and inflammatory responses. NEMO, the NF- $\kappa$ B essential modulator, controls activation of the canonical IKK complex and NF- $\kappa$ B-mediated cellular responses, but details of how this is achieved are not fully known. Our results show that C-terminal mutations in NEMO can cause hyperactivation of inflammatory responses to Toll-like receptor and TNF ligands through impaired recruitment of the negative NF- $\kappa$ B regulator A20/TNFAIP3. Our results help to explain the inflammatory symptoms in patients harboring these NEMO mutations. Furthermore, our findings suggest that targeting this molecular interaction by enhancing A20 expression or its recruitment to the NEMO C-terminus may be a therapeutic strategy for human inflammatory disease.

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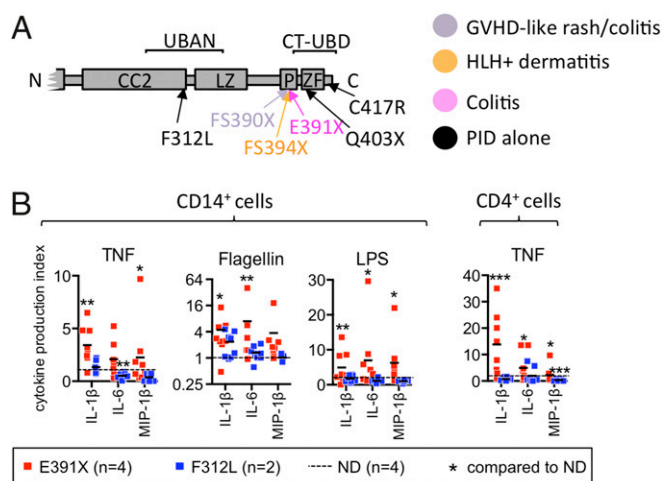
bacterial, viral, or fungal infections, is the salient clinical feature among most individuals diagnosed with EDA-ID. This clinical phenotype can be traced to defects in NF- $\kappa$ B signaling through a number of different receptors families, including TLR, TNF, and antigen receptors. However, inflammatory disease phenotypes, including arthritis, colitis, and graft versus host disease (GVHD)-like dermatitis have also been observed in some patients with NEMO syndrome (15–17). Inflammatory disorders mediated predominantly by cells and molecules of the innate immune system, without evidence of autoantibodies or autoreactive T cells, have been broadly termed “autoinflammatory” (18).

Autoinflammatory manifestations in the NEMO syndrome could result from loss of NF- $\kappa$ B activation and increased cell death, as has been seen in intestinal epithelial cells lacking NEMO (19). NEMO mutations resulting in gain-of-function features may also drive inflammation, although to date none have been described. Here, we report that males harboring mutations leading to truncation of the NEMO C terminus ( $\Delta$ CT-NEMO) develop a distinct syndrome with autoinflammatory disease manifestations, including dermatitis, colitis, arthritis, and macrophage activation syndrome. Remarkably, these  $\Delta$ CT-NEMO mutations enhance NF- $\kappa$ B activation both in the resting state and via increased canonical IKK activity in response to receptor activation. We find that these C-terminal NEMO truncations uniquely fail to recruit the A20-negative regulator of NF- $\kappa$ B, leading to stabilization of K63-ubiquitinated RIP in the TNFR1 signaling complex and enhanced IKK kinase activity. These results represent a novel mechanism of disease pathogenesis, where failure to recruit a negative regulator of inflammatory cytokine signaling contributes to the development of autoinflammatory symptoms in what would otherwise be a primary immunodeficiency syndrome.

## Results

**NEMO C-Terminal Truncation Mutations Associated with Inflammatory Disease Lead to Increased NF- $\kappa$ B-Dependent Responses to Innate Immune Stimuli.** In a genotype-phenotype correlation study, we previously found two “hotspots” of NEMO mutations associated with inflammatory symptoms (20). More detailed analysis of mutations in this region indicated that only nonsense mutations that truncate the C-terminal zinc finger (ZF) domain and adjacent proline-rich sequence, but not missense mutations resulting in single amino acid changes, were associated with inflammatory disease phenotypes (Fig. 1*A* and Table S1). The concordance between the presence of a NEMO truncation and an autoinflammatory phenotype in multiple unrelated individuals suggests that these particular mutations in NEMO, rather than other background genetic or environmental factors, are responsible for the inflammatory disease in these patients. In one large kindred harboring a NEMO C-terminal truncation mutation (E391X), nine individuals, including two females, were affected (16), (Fig. S1*A*, Table S2, and clinical description in *SI Materials and Methods*). In contrast, a kindred with a NEMO-F312L mutation that impairs the ubiquitin binding in ABIN and NEMO (UBAN) domain suffered from typical EDA-ID immunodeficiency symptoms characterized by infection with pneumococcal meningitis, poor antibody response to vaccination, and skin infection with atypical mycobacteria (Fig. S1*A*, Table S2, and clinical description in *SI Materials and Methods*).

To determine the functional consequences of  $\Delta$ CT-NEMO in immune cells, we measured stimulation-induced production of proinflammatory cytokines and chemokines from purified CD14<sup>+</sup> monocytes and CD4<sup>+</sup> T cells isolated from patients and healthy controls. TNF and TLR agonists induced excess IL-1 $\beta$ , IL-6, and MIP-1 $\beta$  in monocytes and CD4<sup>+</sup> T cells with the NEMO-E391X mutation (Fig. 1*B* and Fig. S1*B*). In contrast, cells with the known hypomorphic NEMO-F312L mutation produced similar or reduced cytokines in response to these stimuli compared with healthy donor cells. These changes in cytokine and chemokine production were a result of changes in gene transcription, because TLR stimulation of



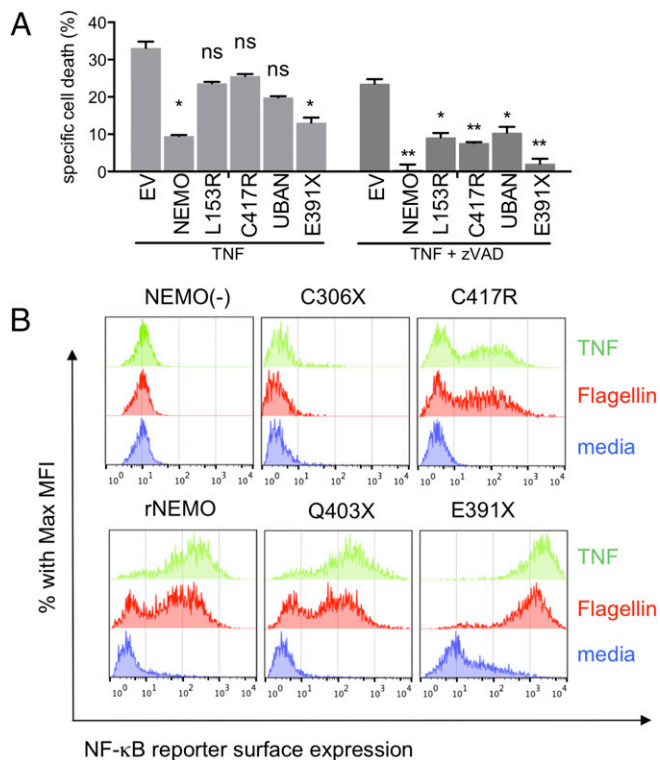
**Fig. 1.** NEMO C-terminal truncation is associated with inflammatory disease and results in enhanced stimulation-induced proinflammatory gene expression and cytokine production in patient monocytes and T cells. (A) NEMO syndrome inflammatory disease phenotypes affecting the C terminus. NEMO-coded protein mutations depict disease phenotypes. Mutations not associated with inflammatory disease but included in this study are shown in black. (B) Healthy control and patient-purified CD14<sup>+</sup> and CD4<sup>+</sup> cells were cultured in media alone or with TNF or TLR ligands for 24 h and secreted cytokines were measured by capture assay. Four individual patients with the NEMO-E391X mutation and two individual patients with the NEMO-F312L mutation were studied. The mean value of three independent experiments (E391X) or two experiments (F312L) is shown by the horizontal black bar. Statistical significance is determined in relation to normal donor. (See Dataset S1 for absolute cytokine production values from patient and healthy control CD14<sup>+</sup> monocytes and CD4<sup>+</sup> T-cell experiments.) GVHD, graft versus host disease; HLH, hemophagocytic lymphohistiocytosis; PID, primary immunodeficiency.

peripheral blood mononuclear cells (PBMC) from individuals with the NEMO-E391X mutation resulted in greater induction of *IL1 $\beta$*  and *IL6* mRNA compared with healthy donor control samples (Fig. S1*C*). As with protein production, the hyperactive response to TLR stimulation was unique to cells from patients with the NEMO C-terminal domain truncation, because NEMO-F312L PBMC exhibited normal *IL1 $\beta$*  and *IL6* expression following stimulation with Flagellin and LPS. These data indicate that unlike all previously described NEMO mutations, the NEMO-E391X mutation confers increased responsiveness to innate immune stimuli.

## NEMO $\Delta$ CT Mutations Potentiate TNFR- and TLR-Induced NF- $\kappa$ B Activity.

The results obtained using primary immune cells ex vivo from patients with NEMO mutations could have been influenced by their clinical status or genetic background. To determine how NEMO-E391X and other  $\Delta$ CT truncations affect NF- $\kappa$ B signaling in a system independent of the effects of EDA-ID, we reconstituted a NEMO-deficient Jurkat T-cell line with physiological levels of wild-type NEMO,  $\Delta$ CT-NEMO, or hypomorphic NEMO mutants using retroviral transduction (20, 21) (Fig. S2*A*). Cells harboring NEMO mutations that fail to activate NF- $\kappa$ B in response to TNF have a characteristic phenotype in which TNF induces apoptosis because of failure to up-regulate NF- $\kappa$ B-dependent anti-apoptotic genes, such as A20 and c-FLIP (20, 22). As previously described, NEMO-deficient Jurkat cells were sensitive to TNF-induced cell death, with both a caspase-dependent and independent component (Fig. 2*A*) (21, 23). Reconstitution of these cells with wild-type NEMO reduced cell death, with the residual cell death being almost completely caspase-dependent, whereas loss of function NEMO mutations affecting the first coiled-coil domain (L153R), the C-terminal zinc finger (C417R), or the UBAN domain (E319AE320A) all failed to prevent cell death in response to TNF (Fig. 2*A*). However, the NEMO-E391X mutant was able to





**Fig. 2.** NEMO-truncation leads to increased NF- $\kappa$ B activity upon TNF-R1 or TLR5 stimulation and prevents TNF-induced apoptosis to the same degree as wild-type NEMO. (A) NEMO-deficient and reconstituted cell lines were cultured in media alone, TNF, or TNF + zVAD for 18 h. Apoptosis was measured by positivity for Annexin-V surface staining and mean percent-specific cell death was calculated as described in *Materials and Methods*, ( $n = 3$ ). (B) Surface protein expression of the NF- $\kappa$ B reporter, rat Thy-1, in NEMO-deficient [NEMO(-)] or reconstituted Jurkat cells was detected by flow cytometry following stimulation in media alone (blue), TNF (green), or Flagellin (red) and quantified ( $n = 3$ ) (Fig. S3A).

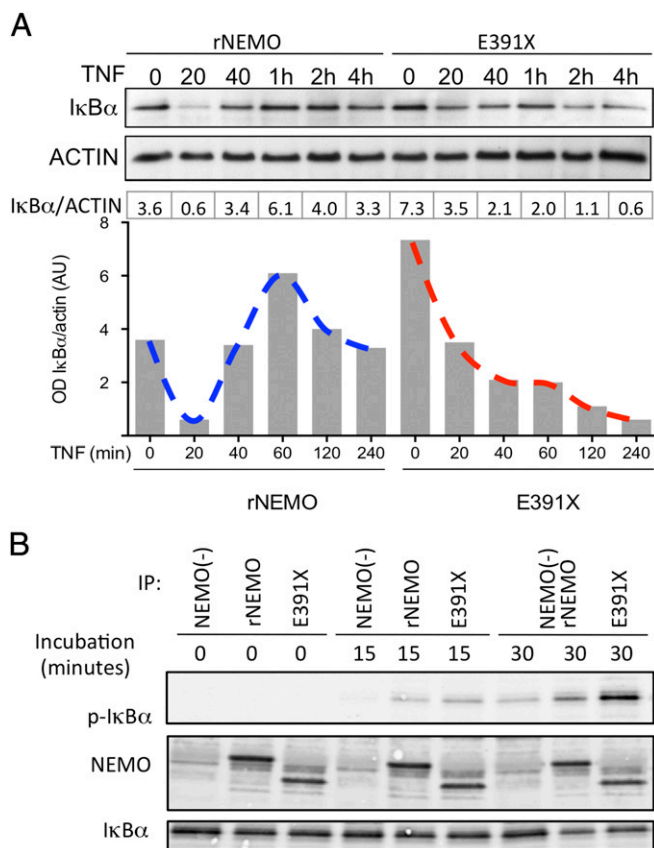
rescue cell death nearly as effectively as wild-type NEMO. These data show that the NEMO-E391X mutant retains ability to promote cell survival following TNF stimulation, suggesting that its NF- $\kappa$ B activating function may be intact.

To more directly measure NF- $\kappa$ B activation induced by  $\Delta$ CT-NEMO mutants, we analyzed reconstituted Jurkat cells by flow cytometry using a stably incorporated NF- $\kappa$ B reporter construct encoding Thy-1. Cells reconstituted with NEMO-C306X, a mutant lacking both ubiquitin-binding domains, were completely unresponsive to either TNF or Flagellin stimulation, similar to NEMO-deficient cells (Fig. 2B). As expected, reconstitution with the previously described patient-derived hypomorphic NEMO C417R led to reduced responsiveness compared with cells reconstituted with wild-type NEMO. In contrast, Jurkat cells expressing the partial  $\Delta$ CT-NEMO mutant Q403X exhibited intact NF- $\kappa$ B responses to TNF and Flagellin, and the full  $\Delta$ CT-NEMO mutant E391X showed substantially increased basal levels of the NF- $\kappa$ B reporter in addition to increased responsiveness compared with cells reconstituted with wild-type NEMO (Fig. 2B, Lower, and Fig. S3A). These results demonstrate  $\Delta$ CT-NEMO mutations preserve NF- $\kappa$ B activity, and in the case of the E391X mutant result in enhanced NF- $\kappa$ B activity compared with full-length NEMO, both in the resting state and in response to TNF or TLR ligands. Taken together, these data show that NEMO-deficient Jurkat T cells reconstituted with  $\Delta$ CT-NEMO mutants recapitulate the increased responsiveness to innate immune stimuli that characterized primary immune cells with these mutants.

**NEMO  $\Delta$ CT Mutants Lead to Sustained IKK Activity and Increased NF- $\kappa$ B Nuclear Translocation.** To determine how  $\Delta$ CT mutants directly affected NF- $\kappa$ B transcription factor activity, we measured NF- $\kappa$ B binding by EMSA following TNF stimulation in NEMO-deficient Jurkat cells reconstituted with wild-type or NEMO-E391X. Extracts from cells expressing NEMO-E391X showed increased binding to NF- $\kappa$ B target sequences following TNF stimulation at all time points (Fig. S3B). Densitometric analysis of NF- $\kappa$ B binding normalized to control Oct-1 binding confirmed this result (Fig. S3B, Right). The E391X mutant specifically activated the canonical NF- $\kappa$ B pathway, as supershift assays showed increased signal shifted with p65 and p50 antibodies but not with RelB, c-Rel or p100 antibodies (Fig. S3C). Increased spontaneous nuclear localization of the p65 subunit of NF- $\kappa$ B was observed in peripheral blood leukocytes from a patient with the E391X-NEMO mutation (Fig. S3D). Similar to what we had observed in the primary peripheral blood cells, mesenchymal stem cell-like fibroblasts generated from patient induced pluripotent stem cells (iPS) displayed constitutive enhanced nuclear NF- $\kappa$ B translocation (Fig. S3E). Taken together, these data show that the NEMO-E391X mutant acts to enhance NF- $\kappa$ B nuclear translocation and DNA binding, both ex vivo from patient cells and in reconstituted cell lines.

To evaluate the mechanisms of enhanced NF- $\kappa$ B signaling in cells expressing  $\Delta$ CT-NEMO mutations in more detail, we used Jurkat cells reconstituted with full-length NEMO and NEMO-E391X. Steady-state levels of I $\kappa$ B $\alpha$  were higher in the E391X mutant compared with wild-type NEMO (Fig. 3A). However, I $\kappa$ B $\alpha$  degradation in the 30 min following TNF stimulation in cells expressing NEMO-E391X appeared equivalent to that in cells with wild-type NEMO (Fig. 3A). Importantly, at longer time points, the I $\kappa$ B $\alpha$  in cells with the E391X-NEMO mutation failed to maintain baseline levels as it does in cells expressing wild-type NEMO, suggesting that sustained IKK kinase activity may be preventing I $\kappa$ B $\alpha$  from reaccumulating. To determine whether C-terminal NEMO truncations increase IKK complex kinase activity, we stimulated activated T cells from EDA-ID patients harboring the  $\Delta$ CT-NEMO-E391X mutation, the NEMO-F312L mutation, which is a loss-of-function missense mutation affecting the UBAN domain, and healthy controls. As expected, TNF induced IKK activity, as measured by phosphorylation of I $\kappa$ B $\alpha$ , was reduced in T cells from an individual with NEMO-F312L loss-of-function mutation (Fig. S3F). Strikingly, there was enhanced phosphorylation of I $\kappa$ B $\alpha$  in T cells from an individual with the E391X mutation compared with a healthy donor control (Fig. S3F), a finding that was reproduced in NEMO-deficient cells reconstituted with full-length NEMO or E391X-NEMO (Fig. S3G), suggesting that this mutation may confer gain-of-function properties to the canonical IKK kinase.

To investigate the effect of  $\Delta$ CT-NEMO on IKK activity in the absence of proteasome-mediated effects on I $\kappa$ B $\alpha$ , we isolated the IKK complex after TNF stimulation and incubated NEMO immunoprecipitates with recombinant GST-I $\kappa$ B $\alpha$ . Kinase activity, as measured by detection of phosphorylation of GST-I $\kappa$ B $\alpha$ , was elevated in IKK immune complexes containing NEMO-E391X compared with those containing wild-type NEMO (Fig. 3B). Taken together, these results strongly suggest that the E391X-NEMO mutation enhances intrinsic IKK activity and transactivation of NF- $\kappa$ B after stimulation with TNF. In general, it is thought that activation of canonical IKK activity occurs via phosphorylation of T-loop serines on IKK- $\alpha$  and IKK- $\beta$  by upstream kinases (24). To determine whether  $\Delta$ CT-NEMO forms enhanced activation of the canonical IKK by this mechanism, we stimulated reconstituted Jurkat cells with TNF and measured specific phosphorylation of T-loop serines by Western blot. We observed equivalent phosphorylation of both T-loop serines and total phosphoserine of IKK $\beta$  in cells that express E391X-NEMO (Fig. S3H and I) compared with full-length NEMO. This finding suggests that enhanced kinase activity in  $\Delta$ CT-NEMO forms is



**Fig. 3.** NEMO-truncation leads to increased TNF-induced IKK complex activity. (A) Western blot of IκBα following TNF stimulation with actin blot as a loading control. Quantitation of IκBα, normalized to actin, was performed by optical densitometry, below. (B) NEMO-deficient and reconstituted Jurkat cell lines were treated with TNF and an in vitro IKK kinase assay (KA) was performed using immunoprecipitated IKK complexes in the presence of ATP and substrate and Western blot analysis of coimmunoprecipitated phospho-IκBα; results are representative of three independent experiments.

not a result of enhanced activation by upstream kinases, but instead, results from altered function or interaction with a signaling partner “downstream” of IKK T-loop activation.

**ΔCT-NEMO Mutants Fail to Interact with A20.** One way by which absence of the NEMO C terminus could lead to enhanced activation of NF-κB would be if ΔCT-NEMO mutants fail to interact with a negative regulator of NF-κB signaling. A20, the product of the gene *TNFAIP3*, is a ubiquitin-editing enzyme that has a well-recognized role as a negative regulator of NF-κB activation (9, 25, 26). We stimulated Jurkat cells reconstituted with wild-type or E391X-NEMO with TNF and examined NEMO and TNFR1-associated protein complexes through immunoprecipitation. Whereas A20 is recruited to full-length NEMO following TNF stimulation, A20 failed to inducibly associate with E391X-NEMO (Fig. 4A). Impaired recruitment of A20 by E391X is specific, as ΔCT-NEMO was able to associate with CYLD in response to TNF stimulation (Fig. S4A and B). Analysis of the TNFR1 signaling complex following TNF stimulation of these Jurkat lines revealed deficient recruitment of A20 to the TNFR1 receptor complex in NEMO-E391X cells (Fig. 4B). A20 within the TNFR complex facilitates the conversion of K63-linked to K48-linked RIP1, leading to subsequent degradation of RIP1 (9). We therefore investigated whether there was increased high molecular-weight RIP1 in association with the IKK complex in cells that express E391X-NEMO. Indeed, coimmunoprecipitation of RIP1 with NEMO indicated

that 30 min after TNF stimulation, high molecular-weight RIP1 could be detected in association with both full-length and E391X-NEMO. Moreover, probing this immunoprecipitate with antibody specific for K63 linkage indicates increased K63-polyubiquitination of proteins associated with NEMO lacking the C terminus (Fig. 4C, Upper, compare lanes 2 and 4). In cells reconstituted with NEMO-E391X, we observed the persistence of high molecular-weight forms corresponding to ubiquitinated RIP1 in the TNFR1 signaling complex compared with cells expressing wild-type NEMO (Fig. 4D and Fig. S4C), reminiscent of the effect of A20 deficiency on RIP1 in the TNFR1 signaling complex (9). Taken together with the increased K63-ubiquitinated protein in the NEMO IP (Fig. 4C), these data most likely represent K63-ubiquitin modified RIP1 that fails to become deubiquitinated because of reduced recruitment of A20 and its K63-deubiquitination activity. These results provide a molecular mechanism for the enhanced NF-κB activation in cells that express ΔCT-NEMO forms by stabilization of polyubiquitinated RIP1 at the TNFR1 signaling complex, and enhanced IKK complex activity resulting from failure to recruit A20 by the NEMO C terminus.

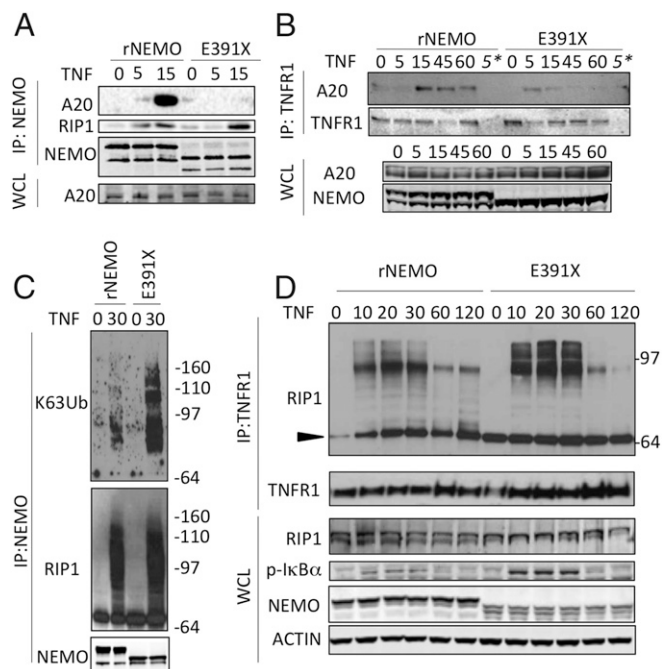
### Discussion

In this study, we have described clinically relevant gain-of-function NEMO mutations, and show that the NEMO C terminus plays an essential regulatory role in NF-κB signaling to prevent inflammatory disease in humans. The impaired ability of these NEMO mutants to interact with a negative regulator of NF-κB, A20, combined with preserved ability to activate the canonical IKK kinase, leads to increased responsiveness to TNF and TLR agonists through a number of mechanisms (Fig. S5). Reduced levels of A20 within the TNFR1 signaling complex results in failure to convert K63 linkages on A20 substrates, such as RIP1 to K48-linked ubiquitin, preserving K63-linked ubiquitin-modified RIP1 and prolonging the recruitment of the IKK signalosome (9).

Our results suggest that the NEMO C terminus plays an essential role in the recruitment and stabilization of A20 to the TNFR. A20 recruitment is in part K63-polyubiquitin-mediated, as the A20 ZnF4 and ZnF7 domains required for inducible recruitment to the TNFR and NEMO, respectively, are K63 ubiquitin binding domains (11, 27, 28). Previous work showed that the NEMO N terminus is required for the formation of the tripartite complex including K63-linked polyubiquitin and A20 (11). Our results demonstrate the essential contribution made by the C terminus of NEMO to A20 recruitment and is consistent with previous work that demonstrates the preferential recognition of K63 linked polyubiquitin by the NEMO C-terminus ubiquitin binding domain (29). Further work needs to be done to understand the details of the biochemical chain of events that lead to A20 recruitment and complex disassembly.

Because ΔCT-NEMO mutants continue to associate with RIP1 (Fig. 4C), our results suggest that other domains within NEMO, such as the UBAN domain that preferentially recognizes linear polyubiquitin, may have increased ability to bind linear ubiquitin in the absence of C-terminus regulation. This could also be mediated through enhanced stabilization of the linear ubiquitin assembly chain complex (LUBAC) complex, which is impaired by the A20 ZF7 domain (25). In addition to preserving RIP1 ubiquitination, failure of A20 recruitment by ΔCT-NEMO forms may also directly enhance canonical IKK activation through loss of the inhibitory effects of A20 following IL-1β, LPS, or TNF stimulation (11, 12). Our results suggest that the C terminus of NEMO is neither required for canonical IKK complex recruitment to the TNFR nor for canonical IKK activation in response to TLR or TNF stimulation. We found that A20 destabilization at the TNFR and impaired recruitment of A20 to ΔCT-NEMO leads to a constitutively active canonical IKK complex that exhibits enhanced kinase activity upon stimulation. These findings are in agreement with prior studies in





**Fig. 4.** Impaired recruitment of A20 to a NEMO C terminus truncation mutant is associated with increased K63-polyubiquitinated RIP1 associated with NEMO and the TNFR. (A) Reconstituted Jurkat T Cells were stimulated with TNF for the indicated times, and NEMO was isolated by immunoprecipitation to detect NEMO/A20 association. NEMO was probed as an immunoprecipitation control. (B) Following TNF stimulation for the indicated times, the TNFR1 was immunoprecipitated (IP) and the associated A20 was detected by Western blot; TNFR1 was probed as a control. The asterisk denotes IP with IgG as a control. (C) Following TNF stimulation, NEMO was immunoprecipitated and the associated RIP1 was detected in addition to specific K63 ubiquitin linkages using K63-linkage-specific antibody, NEMO was probed as an IP control. (D) Following TNF stimulation, TNFR1 was immunoprecipitated and the associated RIP1 was detected by Western blot; unmodified RIP1 is indicated by the black triangle. RIP1, phospho-I $\kappa$ B $\alpha$ , NEMO, and actin were detected in corresponding whole-cell lysates. Experiments were performed in NEMO-deficient Jurkat cells reconstituted with either wild-type or E391X NEMO.

which disrupting the abovementioned A20 ZnF4 or ZnF7 ubiquitin binding domains or the ZnF1 RIP1 interaction domain leads to increased canonical IKK activity (11, 27, 28).

The gain-of-function by  $\Delta$ CT-NEMO mutant forms is in sharp contrast to other NEMO hypomorphic mutations, which result in impaired ability to activate NF- $\kappa$ B (17, 20, 30, 31). Some *IKBK*G mutations can result in the lack of NEMO protein expression; however, impaired canonical IKK activity and NF- $\kappa$ B activation can also be seen in association with mutations with intact protein expression (14, 31). In contrast,  $\Delta$ CT-NEMO is detectable as a truncated protein in whole-cell extracts, and can form a functional canonical IKK complex (Fig. 3B and Figs. S2C and S3 F and G). A number of mechanisms, including enhanced cell death, failure of regulatory immune mechanisms, and infectious triggers may underlie inflammatory disease in the setting of primary immunodeficiency. In patients with  $\Delta$ CT-NEMO mutations, enhanced NF- $\kappa$ B activation leads to proinflammatory cytokine production in response to innate immune stimuli by both CD14<sup>+</sup> monocytes and CD4<sup>+</sup> T cells, which likely accounts for the inflammatory features distinguishing this subset of patients from others with NEMO mutations and EDA-ID.

In addition to the inflammatory disease seen in patients with  $\Delta$ CT-NEMO mutations, some features of immunodeficiency and ectodermal dysplasia persist. Impaired cellular functions leading to immunodeficiency and ectodermal development could stem

from requirements by specific receptors, such as antigen receptors or the EDA receptor for utilization of the C terminus of NEMO to fully activate NF- $\kappa$ B. Similarly, certain specific cell types may require an intact NEMO C terminus to function. Such cell-type and receptor-specific defects have previously been described for the immunodeficiency Mendelian susceptibility to mycobacterial disease (MSMD), that is due to NEMO mutation affecting the UBAN domain and autoinflammatory disease as a result of impaired LUBAC signaling (32, 33). The inflammatory symptoms seen in patients harboring  $\Delta$ CT-NEMO forms are reminiscent of the inflammatory pathology seen in A20-deficient mice that experience arthritis, colitis, and dermatitis (34–36). *TNFAIP3* SNPs that in some cases function to reduce A20 expression levels have been identified that confer susceptibility to rheumatoid arthritis, systemic lupus erythematosus, psoriasis, and Behçets disease (37–40). A recently described familial syndrome with reduced A20 function as a result of haploinsufficiency exhibits inflammatory disease phenotypes that are similar to those with  $\Delta$ CT-NEMO mutation (41). Therefore, our findings underscore the functional importance of A20 in preventing inflammatory disease in humans.

The pathogenesis of inflammatory symptoms in patients with  $\Delta$ CT-NEMO mutations appears to be distinct from the inflammation that can develop in the setting of deficient NF- $\kappa$ B signaling. Mice in which IKK $\beta$  or NEMO was disrupted in colonic epithelial cells develop colitis, presumably because of impaired NF- $\kappa$ B-dependent epithelial cell function and survival during normal homeostasis and infection (19, 42). Similar mechanisms may underlie inflammatory bowel disease in patients with hypomorphic NEMO mutations. LUBAC has recently emerged as an important signaling platform that mediates NF- $\kappa$ B activation under a variety of stimuli. Spontaneous mutation in LUBAC components leads to impaired NF- $\kappa$ B activation, causing chronic dermatitis in mice characterized by inflammatory skin lesions and transient immune cell infiltration of the gut (43, 44). Recently, two kindreds with mutations in HOIL-1 (a LUBAC component) leading to absent protein expression were described (33). Primary fibroblasts from these individuals stimulated with TNF or IL-1 $\beta$  demonstrate impaired NF- $\kappa$ B activation. In contrast, cells harboring  $\Delta$ CT-NEMO mutants have intact activation of NF- $\kappa$ B signaling and transcriptional responses, and are rescued from TNF-induced apoptosis. Unlike LUBAC-, NEMO-, IKK $\beta$ -deficient cells, or cells from the sharpin<sup>cpdm</sup> mutant mouse, inflammation in patients with mutant  $\Delta$ CT NEMO would appear not to result from excessive cell death, but rather selective gain-of-function in activation of the canonical IKK complex. As would be predicted from mouse models in which inflammation resulting from excessive cell death is not confined to the hematopoietic compartment, individuals that express loss-of-function NEMO mutants have experienced residual colitis following hematopoietic stem-cell transplantation (45). In contrast, inflammatory disease symptoms have generally tended to resolve after transplantation in patients with  $\Delta$ CT-NEMO mutations (46), supporting a hematopoietic-cell intrinsic role of immune cell hyperactivation.

The molecular basis of the autoinflammatory phenotype in patients with C-terminal mutations in NEMO represents a novel molecular paradigm. Impaired interaction of NEMO with A20 or other negative regulators of NF- $\kappa$ B may apply to a subset of more common polygenic inflammatory diseases in which NEMO and A20 interaction may be disrupted by other means, such as by altered posttranslational modification of A20 that would impair recruitment to NEMO or decreased expression of A20.

## Materials and Methods

**Informed Consent.** For patient and normal donor-derived peripheral blood samples, informed consent was obtained in accordance with an NIH Institutional Review Board-approved protocol.

### NF- $\kappa$ B Activation and Programmed Cell Death in Reconstituted Jurkat Cells.

Cells were incubated in the presence of TNF 10 ng/mL for 10 h to determine susceptibility to apoptosis, which was evaluated by 7-AAD and AnnexinV-Cy5.5 staining. Parallel cultures were incubated with TNF 10 ng/mL or Flagellin 2  $\mu$ g/mL, and NF- $\kappa$ B-dependent Thy-1 gene transcription was determined by surface staining and flow cytometry (anti-rat Thy1; BD 554898).

**Coimmunoprecipitation and Western Blots.** Cells were lysed in 1% (wt/vol) TritonX-100 containing deubiquitinase inhibitors, and the relevant proteins were detected using specific antibodies. Details can be found in *SI Materials and Methods*.

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