The ubiquitin-editing enzyme A20 requires RNF11 to downregulate NF- κ B signalling

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The RING domain protein RNF11 is overexpressed in breast cancers and promotes tumour growth factor- β (TGF- β) signalling. RNF11 has been proposed to regulate TGF- β signalling by interacting with HECT- and SCF-type E3 ligases; however, the role of RNF11 in other signalling pathways is poorly understood. Here, we demonstrate a novel function of RNF11 as a negative regulator of NF- κ B and jun N-terminal kinase (JNK) signalling pathways. Knockdown of RNF11 with siRNA resulted in persistent tumour necrosis factor (TNF)- and lipopolysaccharide (LPS)-mediated NF- κ B and JNK signalling. RNF11 interacted with the NF- κ B inhibitor A20 and its regulatory protein TAX1BP1 in a stimulus-dependent manner. RNF11 negatively regulated RIP1 and TRAF6 ubiquitination upon stimulation with TNF and LPS, respectively. Furthermore, RNF11 was required for A20 to interact with and inactivate RIP1 to inhibit TNF-mediated NF- κ B activation. Our studies reveal that RNF11, together with TAX1BP1 and Itch, is an essential component of an A20 ubiquitin-editing protein complex that ensures transient activation of inflammatory signalling pathways.

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Introduction

The NF- κ B transcription factor has key functions in innate and adaptive immunity, cell survival and development. NF- κ B is activated in response to a wide range of stimuli, including proinflammatory cytokines, bacterial lipopolysaccharide (LPS) and viral infection (Hayden and Ghosh, 2008). The majority of NF- κ B inducers promotes the activation of a trimeric I κ B kinase (IKK) complex consisting of IKK α , IKK β and IKK γ (also known as NEMO) (Hacker and Karin, 2006). IKK phosphorylates the NF- κ B inhibitory protein I κ B α that normally retains NF- κ B dimers in the cytoplasm, thus leading to I κ B α ubiquitination and degradation by the 26S protea-

some (Karin and Ben-Neriah, 2000). Activation of NF- κ B is tightly regulated to ensure a transient activation response as several NF- κ B target genes such as I κ B α , A20 and CYLD function as inhibitors of NF- κ B in a negative feedback loop (Krikos *et al*, 1992; Sun *et al*, 1993; Jono *et al*, 2004). Because NF- κ B is a critical regulator of proinflammatory genes, persistent NF- κ B activation promotes chronic inflammation, autoimmunity and malignancy (Karin and Greten, 2005).

The zinc-finger protein A20 (also known as TNFAIP3) is a key negative regulator of NF- κ B signalling downstream of innate immune receptors such as tumour necrosis factor receptor (TNFR) and Toll-like receptors (TLRs) (Sun, 2008). A20 exerts deubiquitinating (DUB) activity through an N-terminal catalytic domain of the ovarian tumour (OTU) superfamily (Evans *et al*, 2004; Wertz *et al*, 2004). A20 is transcriptionally induced by NF- κ B and inactivates critical signalling molecules such as RIP1 and TRAF6 in the TNFR and TLR4 pathways (Boone *et al*, 2004). A20 has been proposed to function as a ubiquitin-editing enzyme that first deubiquitinates lysine 63 (K63)-linked polyubiquitin chains on RIP1 and then facilitates the formation of lysine 48 (K48)-linked polyubiquitin chains that lead to RIP1 proteasomal degradation (Wertz *et al*, 2004). A physiological role for A20 was provided by A20-deficient mice that succumbed to massive inflammation and cachexia shortly after birth, indicating a critical role of A20 in restricting NF- κ B and inflammation *in vivo* (Lee *et al*, 2000). Recent studies have indicated that A20 requires the regulatory protein TAX1BP1 (also known as T6BP or TXBP151) and the HECT domain E3 ligase Itch to inhibit NF- κ B and the MAP kinase c-jun N-terminal kinase (JNK) (Shembade *et al*, 2007a, 2008). TAX1BP1 functions as a ubiquitin-binding adaptor (UBA) molecule for A20 as it promotes an interaction between A20 and its substrates RIP1 and TRAF6 (Shembade *et al*, 2007a; Iha *et al*, 2008). The role of Itch in this complex is less clear although it requires an intact HECT domain, suggesting that Itch may be involved in the K48-linked ubiquitination of A20 substrates (Shembade *et al*, 2008). ABIN-1 also interacts with A20 and is required for A20 to deubiquitinate NEMO, suggesting that ABIN-1 is also a component of the A20 ubiquitin-editing complex (Mauro *et al*, 2006).

RING finger protein 11 (RNF11) is a 154 amino-acid protein that was identified as a gene overexpressed in breast cancer (Burger *et al*, 1998; Seki *et al*, 1999). Subsequent studies have revealed that RNF11 is also overexpressed in tumours of the pancreas and colon (Subramaniam *et al*, 2003). RNF11 contains a RING-H2-finger domain in its C terminus that may facilitate protein–protein interactions regulating ubiquitin-mediated proteolysis (Connor and Seth, 2004). RNF11 also has a ‘PPXY’ (where P = proline, X = any amino acid and Y = tyrosine) motif that mediates interactions with WW domain-containing proteins such as the HECT domain E3 ligase Itch (Sudol *et al*, 1995; Kitching *et al*, 2003). RNF11 interacts with components of the ubiquitin pathway, including E2 ubiquitin-conjugating enzymes and E3 ubiquitin

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ligases (Kitching *et al*, 2003; Connor and Seth, 2004). RNF11 has also been demonstrated to modulate tumour growth factor-beta (TGF- β) signalling by interacting with the E3 ligase Smurf2 (Subramaniam *et al*, 2003; Li and Seth, 2004). RNF11 has been proposed to antagonize the degradation of Smurf2 substrates such as the TGF- β receptor (Azmi and Seth, 2005). Furthermore, RNF11 also interacts directly with Smad4 and enhances its trans-activation potential (Azmi and Seth, 2005). Large-scale two-hybrid screening examining protein–protein interactions in the TGF- β pathway using RNF11 as a bait yielded over 50 putative proteins reported to interact with RNF11 (Colland *et al*, 2004). Interestingly, TAX1BP1, A20, Itch, ABIN-1 and NEMO were identified as potential RNF11-binding partners, suggesting that RNF11 may regulate NF- κ B signalling (Colland *et al*, 2004).

Here, we investigated the possibility that RNF11 interacts with and regulates the A20 ubiquitin-editing complex. We found that RNF11 interacted with A20, TAX1BP1 and RIP1 in a TNF-dependent manner. RNF11 inhibited NF- κ B activation in response to TNF and LPS stimulation. Knockdown of RNF11 with siRNA in monocytes resulted in persistent NF- κ B and JNK signalling and enhanced expression of NF- κ B target genes. Furthermore, stimulus-induced RIP1 and TRAF6 polyubiquitination were significantly increased upon siRNA-mediated RNF11 knockdown. We also found that RNF11 was required for A20 recruitment to RIP1 and subsequent inactivation of RIP1 after TNF stimulation. Both the RING domain and PPXY motif were required for RNF11 to interact with A20, TAX1BP1 and RIP1 and to terminate NF- κ B signalling. Thus, RNF11 is a critical regulator of A20 and appears to be a novel component of the A20 ubiquitin-editing complex.

Results

RNF11 interacts with components of the A20 ubiquitin-editing complex

RNF11 has been shown to interact with A20 and known subunits of the A20 ubiquitin-editing complex by yeast two-hybrid screening (Colland *et al*, 2004). However, these interactions have not been confirmed in mammalian cells. Therefore, we first overexpressed RNF11 together with A20, TAX1BP1 and STAT1 (as a negative control) in human embryonic kidney (HEK) 293T cells for co-immunoprecipitation (co-IP) assays. RNF11 interacted with A20 and TAX1BP1, but not STAT1, under overexpression conditions (Figure 1A). Prior studies from our laboratory have demonstrated that endogenous subunits of the A20 ubiquitin-editing complex interact in a manner dependent on TNF stimulation (Shembade *et al*, 2008). Thus, we examined interactions among endogenous RNF11 and A20, TAX1BP1 and RIP1 in the absence and presence of TNF. Immunoprecipitation with RNF11 antibody, but not isotype control, also yielded A20, TAX1BP1 and RIP1, but only after TNF stimulation (Figure 1B). These interactions were also observed in mouse bone marrow-derived macrophages (BMDMs) treated with TNF (Figure 1C). To examine the kinetics of RNF11 binding to the A20 ubiquitin-editing complex, MEFs were stimulated with TNF for various times up to 2 h. As seen in Figure 1D, RNF11 interacted with both TAX1BP1 and A20 transiently as the interactions were lost after 1 h. Interestingly, the interaction between RNF11 and RIP1 was prolonged and was still apparent after 2 h of TNF stimulation (Figure 1D). RNF11 most likely interacted with RIP1 in the cytoplasm as RNF11 was not recruited to the TNFR1 upon

Figure 1 Dynamic interactions between RNF11 and the A20 ubiquitin-editing complex. **(A)** 293T cells were transfected with plasmids encoding Flag-tagged TAX1BP1, A20 and STAT1 in the absence or presence of Myc-RNF11. After 36 h, lysates were immunoprecipitated with anti-Myc followed by immunoblotting with anti-Flag. Lysates were examined for the expression of ectopic proteins by immunoblotting with anti-Flag and Myc. IgH, immunoglobulin heavy chain. **(B)** MEFs were untreated or treated with TNF (10 ng/ml) for 30 min. Cells were lysed in RIPA buffer and immunoprecipitated with anti-RNF11 or isotype control immunoglobulin (control Ig) and immunoblotted with anti-TAX1BP1, A20, RIP1 and RNF11. **(C)** BMDMs or **(D)** MEFs were untreated or treated with TNF (10 ng/ml) for the indicated times. Lysates were immunoprecipitated with anti-RNF11 or control Ig and immunoblotted with anti-TAX1BP1, A20 and RIP1.

TNF stimulation (data not shown). Thus far, we conclude that RNF11 indeed interacts with A20, TAX1BP1 and RIP1 in a stimulus-dependent manner.

RNF11 is a negative regulator of NF- κ B and JNK

We next examined the functional effects of RNF11 on NF- κ B activation. Luciferase assays were performed in THP-1 human monocytic cells using an NF- κ B responsive reporter. Overexpression of RNF11 significantly attenuated TNF-mediated NF- κ B activation (Figure 2A). Conversely, knockdown of endogenous RNF11 with a pool of four distinct siRNA duplexes enhanced TNF-mediated NF- κ B activation compared with a control scrambled siRNA (Figure 2A). We also confirmed the results obtained with the RNF11 siRNA using an independent siRNA duplex targeting a distinct region of RNF11 (Supplementary Figure S1). The effects of RNF11 were specific to NF- κ B as RNF11 knockdown or overexpression had no effect on NFAT activation by PMA and ionomycin stimulation (Figure 2B). Knockdown of endogenous RNF11 and expression of ectopic RNF11 were confirmed by immunoblotting (Figure 2A and B). RNF11 also inhibited LPS-mediated NF- κ B luciferase activation in THP-1 cells (data not shown) and in 293-TLR4/MD2-CD14 cells (Supplementary Figure S2). Finally, IL-1 and poly (I:C)-induced NF- κ B activation were also inhibited by RNF11 overexpression (data not shown).

To determine where in the pathway RNF11 was inhibiting NF- κ B, we performed an epistasis analysis with overexpressed TNFR and TLR4 signalling proteins in 293T cells. RNF11 inhibited NF- κ B activation to varying degrees triggered by TRAF2, RIP1, TRAF6, MyD88, TAB/TAK1 and IKK β (Supplementary Figure S3). However, RNF11 did not inhibit NF- κ B activation induced by p65 overexpression (Supplementary Figure S3). These data suggest that RNF11 inhibits NF- κ B at the level of IKK, although it may also function at additional steps upstream of IKK.

To further examine the effect of RNF11 on NF- κ B activation, we examined the phosphorylation of I κ B α and JNK in THP-1 cells. Cells were transfected with control or RNF11 siRNA and immunoblotting was performed to determine the efficiency of RNF11 knockdown. As seen in Figure 3A, RNF11, but not control scrambled, siRNA led to a significant decrease in the levels of endogenous RNF11 protein. As expected, in the presence of control siRNA, TNF triggered a transient phosphorylation of I κ B α that led to its degradation and resynthesis after 1 h (Figure 3A). Interestingly, knockdown of RNF11 led to a more persistent TNF-mediated I κ B α phosphorylation and degradation (Figure 3A), similar to what has been observed in cells deficient for A20, TAX1BP1 or Itch (Shembade *et al*, 2008). There was also a defect in the termination of TNF-mediated JNK phosphorylation in the presence of RNF11 siRNA (Figure 3A). We next examined the effect of RNF11 siRNA on LPS-mediated activation of NF- κ B and JNK. In the presence of control siRNA, LPS stimulation led to transient I κ B α and JNK phosphorylation as expected (Figure 3B). However, LPS stimulation triggered persistent NF- κ B and JNK signalling when siRNAs for either RNF11 or A20 were transfected (Figure 3B). Therefore, RNF11, similar to A20, appears to have a critical non-redundant function in terminating an NF- κ B response.

We next used real-time PCR to examine the effect of RNF11 knockdown on the expression of A20 and I κ B α , both well-

Figure 2 RNF11 specifically inhibits NF- κ B activation. **(A)** THP-1 cells were transfected on day 1 with no siRNA (mock), control scrambled siRNA or RNF11 siRNA. On day 2, the same cells were transfected with pRL-tk internal control *Renilla* luciferase plasmid, κ B-TATA Luc and Myc-RNF11 as indicated. After an additional 2 days, cells were treated with TNF (10 ng/ml) for 8 h and lysates were subjected to dual luciferase assays. The lysates were also subjected to immunoblotting to examine the expression of RNF11 and β -actin. Error bars indicate s.e.m. of triplicate samples. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer test for multiple comparisons (* $P < 0.001$ compared with control TNF-treated samples). **(B)** THP-1 cells were transfected as described in (A) but NFAT-Luc was included in lieu of NF- κ B Luc. After an additional 2 days, cells were treated with PMA (10 ng/ml) and ionomycin (2 mM) for 8 h for dual luciferase assays. The lysates were also subjected to immunoblotting to examine the expression of RNF11 and β -actin. Error bars indicate s.e.m. of triplicate samples.

characterized NF- κ B target genes (Krikos *et al*, 1992; Sun *et al*, 1993). In the presence of control siRNA, TNF induced the mRNA levels of both A20 and I κ B α , with maximal induction attained after 2 h stimulation (Figure 3C). Remarkably, RNF11 knockdown resulted in a more robust induction of A20 and I κ B α mRNA, particularly at early time points (Figure 3C). We also determined the effect of RNF11 knockdown on the induction of the proinflammatory cytokine IL-6, another NF- κ B target gene. IL-6 protein levels were measured by ELISA using supernatants from THP-1 cells treated with TNF or IL-1. As seen in Figure 3D, significantly increased amounts of IL-6 were detected after cells were

Figure 3 RNF11 is essential for the termination of NF- κ B signalling. (A) THP-1 cells were transfected on consecutive days with control scrambled or RNF11 siRNAs. At 2 days after the second transfection, cells were treated with TNF (10 ng/ml) for the indicated times. Cells were lysed and immunoblotted with anti-I κ B α , pI κ B α , JNK, pJNK and RNF11. (B) THP-1 cells were transfected on consecutive days with control scrambled, RNF11 or A20 siRNAs. At 2 days after the second transfection, cells were treated with LPS (1 μ g/ml) for the indicated times. Cells were lysed and immunoblotted with anti-I κ B α , pI κ B α , JNK, pJNK, A20, RNF11 and β -actin. (C) THP-1 cells were transfected with control scrambled or RNF11 siRNA as described in (A). At 2 days after the second transfection, cells were stimulated with TNF (10 ng/ml) for various times and RNA was subjected to real-time PCR for I κ B α and A20 expression. This experiment was repeated twice with similar results. (D) THP-1 cells were transfected with siRNAs as described in (A). Supernatants were subjected to an IL-6 ELISA. Error bars indicate s.e.m. of triplicate samples. Statistical analysis was performed by one-way ANOVA, followed by the Tukey–Kramer test for multiple comparisons. * P < 0.01 (compared with control IL-1-treated samples); ** P < 0.001 (compared with control TNF-treated samples).

treated with IL-1 or TNF in the presence of RNF11 siRNA. Thus, RNF11 clearly functions as a negative regulator of NF- κ B and is required for maintaining transient NF- κ B and JNK signalling.

RNF11 negatively regulates RIP1 and TRAF6 ubiquitination

As RNF11 negatively regulated IKK and JNK activation (Figure 3) and interacted with RIP1 (Figure 1), we next examined the role of RNF11 in regulating upstream signalling events. Upon TNF binding to TNFR1, RIP1 is recruited to the receptor where Lys 377 serves as an acceptor site for K63-linked polyubiquitin chains (Ea *et al*, 2006). To attenuate TNF signalling, A20 targets RIP1 for inactivation by removing the K63-linked ubiquitin chains and adding K48-linked ubiquitin chains to promote degradation (Wertz *et al*, 2004). Thus, we

examined TNF-induced RIP1 ubiquitination in cells expressing control or RNF11 siRNA. Interestingly, cells transfected with RNF11 siRNA exhibited elevated levels of ubiquitinated RIP1 after TNF treatment compared with control siRNA (Figure 4A). Importantly, similar levels of RIP1 were immunoprecipitated in the presence of both control and RNF11 siRNA (Figure 4A). RNF11 siRNA effectively diminished the expression of endogenous RNF11 protein as revealed by immunoblotting (Figure 4A, lower panel).

TRAF6 is an essential signalling component of IL-1R/TLR4 signalling and is rapidly autoubiquitinated through K63-linked polyubiquitin chains after cells are stimulated with IL-1 or LPS (Chen, 2005). A20 targets TRAF6 for deubiquitination as part of its mechanism to terminate IL-1R/TLR4 signalling (Boone *et al*, 2004). Therefore, we next examined the effect of RNF11 knockdown on LPS-mediated TRAF6

Figure 4 RNF11 negatively regulates the ubiquitination of RIP1 and TRAF6. **(A)** THP-1 cells were transfected on consecutive days with control scrambled or RNF11 siRNAs. At 2 days after the second transfection, cells were treated with TNF (10 ng/ml) for the indicated times. IPs were performed with anti-RIP1 or isotype control (control Ig) and immunoblotted with anti-Ub or RIP1. Lysates were examined for RNF11 expression by immunoblotting with anti-RNF11. Molecular sizes are shown to the left of the Ub blot panel. **(B)** THP-1 cells were transfected with siRNAs as described in (A). Cells were treated with LPS (1 μ g/ml) for the indicated times. IPs were performed with anti-TRAF6 or isotype control (control Ig) and immunoblotted with anti-Ub or TRAF6. Molecular sizes are shown to the left of the Ub blot panel. Lysates were examined for RNF11 expression by immunoblotting with anti-RNF11. NS, nonspecific band; IgH, immunoglobulin heavy chain.

ubiquitination. As seen in Figure 4B, TRAF6 ubiquitination was significantly enhanced upon LPS stimulation when RNF11 was knocked down with siRNA. Similar amounts of TRAF6 were immunoprecipitated in all samples, and RNF11 knockdown was confirmed by immunoblotting (Figure 4B). Collectively, these results indicate that RNF11 antagonizes stimulus-dependent ubiquitination of both RIP1 and TRAF6.

RNF11 is required for A20 to interact with and degrade RIP1

As our results implicate RNF11 as a negative regulator of NF- κ B, and interactions between RNF11, A20, TAX1BP1 and Itch were observed, we hypothesized that RNF11 may be a subunit of the A20 ubiquitin-editing complex. Previously, we have demonstrated that A20 is unable to engage RIP1 after TNF stimulation in the absence of TAX1BP1 or Itch (Shembade *et al*, 2007b, 2008). Thus, we performed co-IPs to determine whether RNF11 knockdown impaired the recruitment of A20 to RIP1. Remarkably, when RNF11 or TAX1BP1 expression was silenced by siRNA, A20 was completely impaired in the recruitment to RIP1 (Figure 5A). Both RNF11 and TAX1BP1 were effectively silenced with the siRNA as confirmed by immunoblotting (Figure 5A). Similar results were obtained when examining TNF-induced interaction of Itch and RIP1 (Figure 5B), suggesting that RNF11 is

required for the recruitment of both A20 and Itch to RIP1. As we have demonstrated in Figure 1 that RNF11 is recruited to RIP1 in a TNF-dependent manner, we hypothesized that TAX1BP1 and/or Itch were required for RNF11 to interact with RIP1. Thus, we examined the interaction of RNF11 and RIP1 in *Itch*^{-/-} and *Tax1bp1*^{-/-} MEFs stimulated with TNF. In control MEFs, TNF promoted an interaction between RNF11 and RIP1 (Figure 5C and D). However, in the absence of Itch or TAX1BP1, RNF11 was impaired in binding to RIP1 after TNF treatment (Figure 5C and D). Therefore, each of the molecules in the A20 ubiquitin-editing complex appears to serve essential and non-redundant roles in the assembly of the complex and subsequent recruitment to substrates.

We next examined the role of RNF11 in the inhibition of NF- κ B signalling by A20. First, the effect of RNF11 on the DUB function of A20 was explored by performing TRAF6 ubiquitination assays. As expected, A20 potently inhibited endogenous TRAF6 polyubiquitination in LPS-treated MEFs when control siRNA was transfected (Figure 5E). However, when cells were transfected with RNF11 siRNA, A20-mediated deubiquitination of TRAF6 was completely impaired (Figure 5E). Overexpression of A20, but not an enzymatically inactive form of A20 (A20 C103A), also potently inhibited TNF-mediated activation of an NF- κ B luciferase reporter (Figure 5F). When RNF11 expression was silenced with siRNA, A20 was functionally defective in the inhibition of NF- κ B (Figure 5F).

A20 has also been shown to promote the degradation of RIP1 through K48-linked polyubiquitin chains (Wertz *et al*, 2004). To determine whether A20 required RNF11 to promote the degradation of RIP1, we performed cycloheximide (CHX) chase assays. As expected, A20 triggered the degradation of endogenous RIP1 in MEFs in the presence of control siRNA (Figure 5G). However, when RNF11 expression was silenced with siRNA, A20 was impaired in the degradation of RIP1 (Figure 5G). Taken together, these data suggest that RNF11 is indispensable for A20 recruitment and inactivation of key signalling molecules in the TNFR and TLR4/IL-1R pathways.

The RING domain and PPXY motif of RNF11 are required for binding to A20, TAX1BP1 and RIP1 and terminating NF- κ B signalling

RNF11 has been proposed to exert its function in TGF- β signalling through its RING domain and PPXY motif (Azmi and Seth, 2005). Therefore, we next examined whether these domains had a function in the termination of NF- κ B signalling. We knocked down the expression of endogenous RNF11 with siRNA and reconstituted cells with siRNA-resistant forms of RNF11. THP-1 cells were transfected with control or a single RNF11 duplex siRNA followed by subsequent transfection with siRNA-resistant wild-type Flag-RNF11 (WT^R), Flag-RNF11 C99A (cysteine $\rightarrow$ alanine; C99A^R) with a disrupted RING domain, or Flag-RNF11 Y40A (tyrosine $\rightarrow$ alanine; Y40A^R) with a mutated PPXY motif. Cells were then stimulated with TNF for various times and NF- κ B and JNK activation were monitored by immunoblotting with phospho-specific antibodies. Knockdown of endogenous RNF11 and expression of Flag-tagged RNF11 proteins were confirmed by immunoblotting (Figure 6B). As expected, knockdown of RNF11 promoted the enhanced activation of NF- κ B as revealed by persistent TNF-mediated phosphorylation and degradation of I κ B α (Figure 6B). JNK phosphorylation was

Figure 5 RNF11 is essential for A20 to interact with and inactivate RIP1. (A) THP-1 cells were transfected on consecutive days with control scrambled, RNF11 or TAX1BP1 siRNAs. At 2 days after the second transfection, cells were treated with TNF (10 ng/ml) for 30 min. IPs were performed with anti-RIP1 or isotype control (cont. Ig) followed by immunoblotting with anti-A20 or RIP1. Lysates were immunoblotted with anti-RNF11, TAX1BP1 and β -actin. (B) THP-1 cells were transfected on consecutive days with control scrambled or RNF11 siRNAs. At 2 days after the second transfection, cells were treated with TNF (10 ng/ml) for 30 min where indicated. IPs were performed with anti-Itch or isotype control (cont. Ig) followed by immunoblotting with anti-RIP1 or Itch. Lysates were immunoblotted with anti-RNF11 and β -actin. (C) *Itch*^{+/+} and *Itch*^{-/-} MEFs were treated with TNF (10 ng/ml) for 30 min as indicated. Cells were lysed and immunoprecipitated with anti-RNF11 or isotype control (cont. Ig) followed by immunoblotting with anti-RIP1 or RNF11. (D) *Tax1bp1*^{+/+} and *Tax1bp1*^{-/-} MEFs were treated and subjected to co-IPs as in (C). (E) MEFs were transfected with control scrambled or RNF11 siRNAs. The following day, the cells were transfected with 0.25, 0.5 or 1 μ g of Flag A20 (wedges in lanes 3–5 and 6–8). After 2 days, the cells were lysed and endogenous TRAF6 was immunoprecipitated with anti-TRAF6 followed by immunoblotting with anti-Ub. Molecular sizes are shown to the left of the Ub blot panel. The expression of ectopic A20 was examined by immunoblotting with anti-Flag. The efficiency of RNF11 knockdown was determined by immunoblotting with anti-RNF11. (F) 293T cells were transfected with control scrambled or RNF11 siRNAs. The following day, the cells were transfected with pRL-tk, κ B-TATA-Luc and 0, 0.25, 0.5 or 1 μ g of Flag-A20 or Flag-A20 C103A as indicated (wedges). At 2 days after the second transfection, cells were treated with TNF (10 ng/ml) for 8 h and lysates were subjected to dual luciferase assays. Error bars indicate s.e.m. of triplicate samples. (G) MEFs were transfected with control scrambled or RNF11 siRNAs. The following day, the cells were transfected with 1 μ g of empty vector (lanes 1–4) or Flag A20 (lanes 5–12). At 2 days after the second transfection, cells were treated with CHX for the indicated times. Cells were lysed and the stability of endogenous RIP1 was examined by immunoblotting with anti-RIP1. Lysates were also immunoblotted with anti-Flag, RNF11 and β -actin.

Figure 6 RNF11 requires the RING domain and PPXY motif to interact with the A20 ubiquitin-editing complex and to terminate NF- κ B signalling. (A) Schematic diagram of RNF11 domain organization and mutants used in this figure. The PPXY motif is located between amino acids 37 and 40. The RING finger domain is located between amino acids 99 and 140. The RNF11 C99A mutant has a non-functional RING domain. The RNF11 Y40A mutant has a disrupted PPXY motif. (B) THP-1 cells were transfected with a control scrambled or a single duplex RNF11 siRNA. The following day, the cells were transfected with siRNA-resistant forms of Flag-RNF11 (WT^R), Flag-RNF11 C99A (C99A^R) and Flag-RNF11 Y40A (Y40A^R). After two more days, cells were treated with TNF (10 ng/ml) for the indicated times and lysates were immunoblotted with anti-pI κ B α , I κ B α , pJNK, JNK, Flag, RNF11 and β -actin. (C) THP-1 cells were transfected with siRNA and siRNA-resistant forms of RNF11 as described in (B). At 2 days after DNA transfections, cells were treated with TNF for 30 min where indicated. Lysates were subjected to immunoprecipitations with anti-RNF11 followed by immunoblotting with A20, TAX1BP1 and RIP1. The lysates were also used for immunoblotting using anti-I κ B α , Flag, RNF11 and β -actin.

similarly prolonged when RNF11 expression was silenced with siRNA (Figure 6B). Reconstitution of cells with wild-type Flag-RNF11 (WT^R), but not the RNF11 RING (C99A^R) or PPXY (Y40A^R) mutant, restored transient I κ B α and JNK phosphorylation (Figure 6B). Therefore, these data indicate that RNF11 requires both the RING domain and PPXY motif to terminate TNF-mediated NF- κ B and JNK activation.

A critical step in the termination of NF- κ B signalling is the assembly of the A20 ubiquitin-editing complex in response to TNF or LPS stimulation (Shembade *et al*, 2008). As RING domains and PPXY motifs may regulate protein-protein interactions, we next examined whether the RNF11 RING domain and PPXY motif were essential for assembly of the A20 ubiquitin-editing complex. Cells were transfected with a control siRNA or a single RNF11 siRNA duplex and reconstituted with siRNA-resistant forms of RNF11 as described above. Cells were stimulated with TNF and the interactions

between RNF11, A20, TAX1BP1 and RIP1 were evaluated by co-IPs. As expected, in the presence of control siRNA, RNF11 interacted with A20, TAX1BP1 and RIP1 upon TNF stimulation (Figure 6C). Knockdown of RNF11 impaired these interactions as expected but was restored by the expression of an siRNA-resistant wild-type RNF11 (WT^R; Figure 6C). However, reconstitution with siRNA-resistant RNF11 mutants C99A^R or Y40A^R was unable to restore interactions of RNF11 with A20, TAX1BP1 and RIP1 (Figure 6C). Therefore, we conclude that both the RING domain and PPXY motif are important for stimulus-dependent interactions of RNF11 with TAX1BP1, A20 and RIP1.

Discussion

The A20 ubiquitin-editing complex consisting of A20, TAX1BP1, Itch and ABIN-1 has essential functions in limiting

cytokine-induced NF- κ B activation and inflammation. TAX1BP1 interacts with RIP1 and recognizes K63-linked polyubiquitin chains through a C-terminal zinc-finger domain (Iha *et al*, 2008). Therefore, TAX1BP1 most likely functions as a UBA that connects A20 with ubiquitinated substrates. The A20-binding protein Ymer also interacts with K63-linked polyubiquitin chains and may perform similar functions (Bohgaki *et al*, 2008). Itch is recruited to TAX1BP1 through two PPXY motifs and is required for A20 to inactivate signalling molecules, possibly by facilitating the ubiquitination and degradation of RIP1 (Shembade *et al*, 2008). ABIN-1 is an A20-binding protein that was reported to regulate A20-mediated deubiquitination of NEMO (Mauro *et al*, 2006). Yeast two-hybrid screening identified TAX1BP1, A20, Itch and ABIN-1 as putative binding partners for RNF11 (Colland *et al*, 2004). Furthermore, RNF11 has been demonstrated to interact with Itch in an independent study (Kitching *et al*, 2003). Here, we demonstrated that endogenous RNF11 interacted with TAX1BP1 and A20 in a stimulus-dependent manner. Knockdown of RNF11 with siRNA led to persistent NF- κ B activation and enhanced induction of NF- κ B target genes. Furthermore, RNF11 was required for A20 to interact with and inactivate RIP1. These findings indicate that RNF11 is an essential regulator of A20 and therefore it is most likely a novel subunit of the A20 ubiquitin-editing complex.

RNF11 was shown previously to interact with E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases, indicating a potential regulatory role in protein ubiquitination (Connor and Seth, 2004). RNF11 contains a RING domain that may facilitate protein-protein interactions leading to ubiquitination and degradation of protein substrates (Seki *et al*, 1999). In addition, RNF11 harbours a PPXY motif that is important for binding to WW domain proteins such as the E3 ligases Itch, NEDD4 and Smurf2 (Kitching *et al*, 2003; Subramaniam *et al*, 2003). Our results clearly support a role for both the RNF11 RING domain and PPXY motif in the assembly of the A20 ubiquitin-editing complex and the termination of inflammatory signalling pathways. It is most likely that RNF11 interacts directly with Itch in the A20 ubiquitin-editing complex through the PPXY motif, thus RNF11 may be recruited to TAX1BP1 and A20 through interactions with Itch. Nevertheless, RNF11 has an indispensable function in the recruitment of Itch and A20 to RIP1 upon TNF stimulation. As RNF11 is unable to engage RIP1 in *Itch*^{-/-} and *Tax1bp1*^{-/-} MEFs, it is most likely that each of the molecules in the A20 ubiquitin-editing complex serves non-redundant roles for TNF-dependent binding and subsequent inactivation of RIP1.

We have previously reported that the HECT domain of Itch is pivotal for inhibition of NF- κ B (Shembade *et al*, 2008). This raises an important question as to why two E3 ligases, Itch and RNF11, are required for A20 to inhibit NF- κ B. It is possible that RNF11 and Itch ubiquitinate distinct substrates essential for terminating NF- κ B and JNK signalling. As RNF11 binding to RIP1 was maintained at later time points of TNF stimulation when RNF11 was no longer bound to TAX1BP1 and A20 (Figure 1D), RNF11 may be involved in the K48-linked polyubiquitination of RIP1. Itch may cooperate with RNF11 in this regard or it may regulate another component of the A20 ubiquitin-editing complex through ubiquitination as there is precedence for Itch-mediated K63-linked ubiquitination of substrates (Lee *et al*, 2008). Regardless, we speculate that RNF11 may serve as a regulatory protein for Itch in a

similar capacity as Ndfip1, which promotes Itch function during T-cell activation (Oliver *et al*, 2006). However, RNF11 and Ndfip1 may control the specificity of Itch for substrates in distinct signalling pathways. Future studies will be necessary to determine the precise roles of Itch and RNF11 within the A20 ubiquitin-editing complex.

RNF11 was first isolated as a cDNA overexpressed in breast cancer (Burger *et al*, 1998). Interestingly, A20 expression is also upregulated in other cancers and has been shown to have an important survival function in breast cancer (Vendrell *et al*, 2007). Furthermore, TAX1BP1 is known as an antiapoptotic protein that is cleaved by specific caspases during TNF- and Fas-mediated apoptosis (De Valck *et al*, 1999). Thus, it is intriguing to speculate that A20, TAX1BP1 and RNF11 may function cooperatively to inhibit cell death in certain tumours. As NF- κ B is generally considered antiapoptotic, it may be counterintuitive to consider these NF- κ B inhibitors as antiapoptotic molecules. However, the A20 ubiquitin-editing complex may regulate cell survival independently of NF- κ B. Furthermore, certain cancers have acquired mechanisms to activate NF- κ B that are insensitive to negative regulation by A20, thus explaining the persistent NF- κ B activation in these cells. For example, the NF- κ B-inducing kinase (NIK) is overexpressed in adult T-cell leukaemia, melanoma and Hodgkin's Reed-Sternberg cells and promotes persistent NF- κ B activation that cannot be blocked by A20 (Dhawan and Richmond, 2002; Saitoh *et al*, 2008).

TGF- β has important functions in autoimmunity and T-cell tolerance by downregulating the function of effector T cells and promoting the generation of T regulatory cells (Li and Flavell, 2008). NF- κ B is critical for inflammatory responses by regulating the expression of genes such as cytokines and chemokines that drive inflammation (Hayden and Ghosh, 2008). Our findings coupled with other studies on the role of RNF11 in TGF- β signalling clearly indicate that RNF11 enhances TGF- β signalling, whereas opposing NF- κ B. Therefore, RNF11 is most likely to function as a key negative regulator of inflammatory responses *in vivo* by targeting two critical pathways that control inflammation. RNF11 has recently been shown to be expressed in the brain and is a strong candidate gene for Parkinson's disease (Anderson *et al*, 2007). It is possible that RNF11 may be particularly important in immune-privileged tissues such as the brain that cannot tolerate high levels of inflammation. Conditional *Rnf11* knockout mice will be instrumental in determining the tissue-specific roles of RNF11 in limiting inflammation *in vivo*.

Materials and methods

Cells and biological reagents

THP-1 and 293T cells were purchased from ATCC. 293-TLR4/MD2-CD14 cells were a kind gift from K Tolba (University of Miami). *Itch*^{-/-} and *Tax1bp1*^{-/-} MEFs were described earlier (Shembade *et al*, 2008). THP-1 cells were cultured in RPMI medium with standard formulations. MEFs and 293T cells were cultured in DMEM medium with standard formulations. 293-TLR4/MD2-CD14 cells were grown in DMEM medium supplemented with 10 μ g/ml blasticidin (Invivogen) and 50 μ g/ml hygromycin B (Clontech). Flag-TAX1BP1, Flag-TRAF6, Flag-RIP1, Flag-A20, Flag-A20 C103A and κ B-Luc were described earlier (Shembade *et al*, 2007a). NFAT Luc was purchased from BD Biosciences/Clontech. Flag-TRAF2 was provided by Z Ronai (Burnham Institute). The p65 and IKK β expression vectors were provided by SC Sun (University of Texas MD Anderson). The Myd88 plasmid was provided by G Barber (University of Miami). TAB and TAK1 expression plasmids

were provided by K Tolba (University of Miami). Flag-Stat1 was a kind gift from J Darnell (Rockefeller University). A human RNF11 expression plasmid was purchased from Genscript and used as a template for PCR-mediated cloning of *RNF11* into pCDNA-Myc and p3xFlag vectors. The following antibodies were used in this study: I κ B α (C-21; Santa Cruz Biotechnology), TRAF6 (H-274; Santa Cruz Biotechnology), β -actin (AC-15; Abcam), TAX1BP1 (ab22049; Abcam), JNK (56G8; Cell Signaling), phospho-JNK (9251; Cell Signaling), phospho-I κ B α (14D4; Cell Signaling), A20 (clone E5-1619; BD Biosciences Pharmingen), Itch (clone 32; BD Biosciences Pharmingen), RIP1 (clone 38; BD Biosciences Pharmingen), Flag (M2; Sigma), HA (12CA5; Roche), RNF11 (A01; Abnova) and Ubiquitin (SPA-200F; Assay Designs). Recombinant TNF and IL-1 were purchased from R&D Systems. CHX and LPS were purchased from Sigma.

Isolation of BMDMs

BMDMs were isolated using a standard protocol (Waterfield *et al*, 2003). Bone marrow cells were obtained from the femurs of C57BL/6 mice and were cultured in L929 cell-conditioned media for 7 days prior to TNF stimulation. The macrophages were highly pure (> 98%) as determined by anti-CD11b staining and flow cytometry. Mice were housed under specific pathogen-free conditions and experiments were in accordance with institutional guidelines and approved by the animal care and use committee at the University of Miami.

RNA interference

Control scrambled, A20 (TNFAIP3) and RNF11 SMARTpool siGenome siRNAs were purchased from Dharmacon. Control scrambled, TAX1BP1 siRNA (no. 138154) and an independent RNF11 siRNA (Silencer select RNF11 siRNA no. s25670) were purchased from Ambion. Unless indicated, all siRNA studies were performed with SMARTpool siGenome siRNAs. For RNF11 knock-down-reconstitution experiments in THP-1 cells, an individual RNF11 siRNA duplex was purchased from Dharmacon. The sense sequence is: 5'-UAGGAUAGCUCAAAGAAUAAU-3'.

Site-directed mutagenesis

Site-directed mutagenesis of RNF11 (Y40A and C99A) was performed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer's instructions. The siRNA-resistant Flag-RNF11 plasmids were constructed using primers to introduce silent mutations in amino acids Q72 and R73 in RNF11 without altering the coding sequence. Primer sequences are available upon request.

Transfections and luciferase assays

Transient transfections in 293T cells were performed using calcium phosphate according to a standard protocol. THP-1 cells were transfected with LipofectamineTM LTX (Invitrogen) according to the manufacturer's instructions. 293-TLR4/MD2-CD14 cells and MEFs were transfected with FuGENE[®] 6 or FuGENE[®] HD (Roche) according to the manufacturer's instructions. For siRNA transfections, cells were transfected with 60 pmol of siRNA on consecutive days using LipofectamineTM Plus or Lipofectamine LTX (Invitrogen). For luciferase assays, cells were harvested 36–48 h after the last transfection and cell lysates were prepared in 1 $\times$ Passive Lysis Buffer (Promega). Luciferase activity was assayed using the Dual Luciferase Assay system according to the manufacturer's instructions (Promega). Firefly luciferase values were normalized based on

the *Renilla* luciferase internal control values. Luciferase values are presented as 'fold induction' relative to the untreated control transfected with either empty vector or control siRNA.

ELISA

The IL-6 ELISA was performed according to the manufacturer's instructions (eBioscience). Supernatant from TNF- and IL-1-stimulated THP-1 cells was used for the ELISA. The amount of IL-6 was calculated from a standard curve derived from recombinant IL-6.

Real-time PCR

Real-time PCR was performed essentially as described (Harhaj *et al*, 2007a). Total RNA was isolated from cells using the RNeasy mini kit (Qiagen). RNA was converted to cDNA using the first strand cDNA synthesis kit for RT-PCR (AMV; Roche). Real-time PCR was performed on a LightCycler[®] instrument (Roche) using LightCycler FastStart DNA Master^{PLUS} HybProbe (Roche) and gene-specific TaqMan probes (Applied Biosystems). Gene expression for A20 and I κ B α was normalized to the internal control 18S rRNA.

CHX chase assays

CHX chase assays were performed as described earlier (Shembade *et al*, 2008). Cells were treated with CHX (100 μ g/ml) for various times 2 days after transfections. Cells were lysed in RIPA buffer and the stability of endogenous RIP1 was analysed by immunoblotting.

Co-IP and ubiquitination assays

Co-IP and ubiquitination assays were performed as described earlier (Xiao *et al*, 2001; Shembade *et al*, 2007a). Briefly, transfected 293T, MEFs or THP-1 cells were lysed in RIPA buffer and immunoprecipitated with specific antibodies. Immunoprecipitates were washed three times with RIPA buffer followed by a wash with RIPA containing 1 M urea for ubiquitination assays. Immunoblotting was performed with the indicated antibodies for either co-IPs or ubiquitination assays.

Immunoblotting

Immunoblotting was performed as described earlier (Harhaj *et al*, 2007b; Shembade *et al*, 2007b). Whole-cell lysates were resolved by SDS-PAGE, transferred to nitrocellulose membranes, blocked in 5% milk, incubated with the indicated primary and secondary antibodies and then detected with Western Lightning Enhanced Chemiluminescence reagent (Perkin Elmer).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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