A20 inhibits LUBAC-mediated NF- κ B activation by binding linear polyubiquitin chains via its zinc finger 7

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Linear polyubiquitination of proteins has recently been implicated in NF- κ B signalling and is mediated by the linear ubiquitin chain assembly complex (LUBAC), consisting of HOIL-1, HOIP and Sharpin. However, the mechanisms that regulate linear ubiquitination are still unknown. Here, we show that A20 is rapidly recruited to NEMO and LUBAC upon TNF stimulation and that A20 inhibits LUBAC-induced NF- κ B activation via its C-terminal zinc-finger 7 (ZF7) domain. Expression of a polypeptide corresponding to only ZF7 was sufficient to inhibit TNF-induced NF- κ B activation. Both A20 and ZF7 can form a complex with NEMO and LUBAC, and are able to prevent the TNF-induced binding of NEMO to LUBAC. Finally, we show that ZF7 preferentially binds linear polyubiquitin chains *in vitro*, indicating A20–ZF7 as a novel linear ubiquitin-binding domain (LUBID). We thus propose a model in which A20 inhibits TNF- and LUBAC-induced NF- κ B signalling by binding to linear polyubiquitin chains via its seventh zinc finger, which prevents the TNF-induced interaction between LUBAC and NEMO.

The EMBO Journal (2012) 31, 3845–3855. doi:10.1038/emboj.2012.240; Published online 28 August 2012

Subject Categories: signal transduction; proteins

Keywords: A20; NF- κ B; TNFAIP3; ubiquitin; zinc finger

Introduction

The transcription factor nuclear factor κ B (NF- κ B) is involved in the regulation of diverse biological processes, such as inflammation, development, innate and adaptive immunity, cell proliferation and survival. The activation of NF- κ B is mainly regulated by different types of polyubiquitination, which differ by the use of distinct lysine residues of ubiquitin to form the polyubiquitin chain, and has been best described

for TNF-induced NF- κ B signalling (Grabbe *et al*, 2011). K48-linked polyubiquitination of the NF- κ B inhibitory protein I κ B α by the SCF- β TrCP E3 ubiquitin ligase triggers its subsequent degradation by the proteasome, allowing the nuclear translocation of the NF- κ B dimer. On the other hand, cIAP-mediated K63-linked polyubiquitination of specific NF- κ B signalling proteins such as RIP1 does not trigger their degradation but is recognized by other proteins via specific ubiquitin-binding domains and mediates specific protein–protein interactions that are crucial for NF- κ B signalling. Linear polyubiquitination is the most recently described type of polyubiquitination with a role in TNF-induced NF- κ B signalling (Haas *et al*, 2009; Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011; Verhelst *et al*, 2011), and is characterized by binding of the α -amino group of Met1 of the proximal ubiquitin moiety to the C-terminal Gly76 of the distal ubiquitin moiety. The only E3 ligase complex known so far to generate linear ubiquitin chains is the 600-kDa linear ubiquitin chain assembly complex (LUBAC) (Kirisako *et al*, 2006). The LUBAC complex consists of a catalytic subunit HOIP (HOIL-1-interacting protein; also known as RNF31) and the two regulatory subunits HOIL-1 (haem-oxidized iron-regulatory protein 2 ubiquitin ligase-1; also known as RBCK1) and Sharpin (SHANK-associated RH domain-interacting protein), which share high sequence similarity and interact with HOIP through their respective ubiquitin-like (UBL) domains. Although both HOIP and HOIL-1 contain the catalytic RING between RING (RBR) domains, only the RBR domain of HOIP is essential for NF- κ B signalling. The actual stoichiometry of the LUBAC complex has not been resolved and besides a heterotrimeric HOIL-1/HOIP/Sharpin complex also a heterodimeric HOIP/Sharpin complex can conjugate linear chains on NEMO (Gerlach *et al*, 2011; Ikeda *et al*, 2011; Tokunaga *et al*, 2011). However, cells defective in one of the LUBAC components already show reduced NF- κ B activation and increased apoptosis in response to TNF (Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011). Upon TNF stimulation, LUBAC is recruited to the TNF-R signalling complex where it modifies the I κ B kinase (IKK) adaptor protein NEMO, and to a lesser extent RIP1, with linear polyubiquitin chains (Haas *et al*, 2009; Tokunaga *et al*, 2009; Gerlach *et al*, 2011). The conjugated linear chains are specifically recognized by NEMO, HOIL-1 and Sharpin (Rahighi *et al*, 2009; Gerlach *et al*, 2011; Sato *et al*, 2011), which is believed to further stabilize the TNF-R1/NEMO/LUBAC signalling complex and recruit additional NEMO molecules, facilitating IKK activation and NF- κ B-dependent gene expression. The NZF (Npl4 zinc-finger) domain of HOIL-1 and Sharpin, and the UBA1 (ubiquitin-binding domain in ABIN and NEMO) domain are the only ubiquitin-binding domains known to preferentially bind linear chains (Wagner *et al*, 2008; Sato *et al*, 2011; Kensche *et al*, 2012).

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Received: 19 February 2012; accepted: 3 August 2012; published online: 28 August 2012

Several negative regulatory mechanisms keep NF- κ B signalling in check. In this context, the NF- κ B inhibitory protein A20 (also known as TNFAIP3) plays a key role in the negative regulation of TNF and Toll-like receptor responses (Coornaert *et al*, 2009). A20 expression is upregulated by NF- κ B activation and A20 acts in a negative feedback loop to control NF- κ B-dependent gene expression. A20-deficient mice are hyperresponsive to TNF and LPS, develop multi-organ inflammation, and die within 2 weeks of birth (Lee *et al*, 2000). Mice lacking A20 in specific cell types develop arthritis, colitis, systemic lupus erythematosus and other autoimmune symptoms, further illustrating that A20 clearly has an important role in many different cell types (Tavares *et al*, 2010; Vereecke *et al*, 2010; Chu *et al*, 2011; Hammer *et al*, 2011; Hovelmeyer *et al*, 2011; Kool *et al*, 2011; Matmati *et al*, 2011). Furthermore, the *TNFAIP3* gene has been identified as a susceptibility locus for several human inflammatory and autoimmune diseases, including inflammatory bowel disease, rheumatoid arthritis, psoriasis, lupus and type 1 diabetes (Vereecke *et al*, 2009). A20 is also frequently inactivated in subsets of B-lineage lymphomas that are characterized by NF- κ B hyperactivation and A20 has therefore been suggested to be a novel tumour suppressor (Honma *et al*, 2009; Kato *et al*, 2009; Schmitz *et al*, 2009).

A20 is believed to inhibit NF- κ B with the help of its ubiquitin-editing functions (Wertz *et al*, 2004). The N-terminal half of A20 encodes a deubiquitinating (DUB) domain that mediates the deubiquitination of K63-polyubiquitinated NF- κ B signalling proteins such as TRAF6 and RIP1 (Boone *et al*, 2004; Wertz *et al*, 2004). The C-terminal half of A20 encodes seven zinc-finger (ZF) motifs that have been shown to confer E3 ubiquitin ligase activity to A20. In the TNF signalling pathway, removal of K63-linked polyubiquitin chains from RIP1 is followed by its A20-mediated K48-polyubiquitination, promoting its proteasome mediated degradation and the termination of pro-inflammatory pathways (Wertz *et al*, 2004). Furthermore, A20 can indirectly affect the ubiquitination status of signalling proteins by preventing the interaction between E2 ubiquitin conjugating enzymes and specific E3 ubiquitin ligases, such as Ubc13/TRAF2 or Ubc5/TRAF6, via competitive binding and followed by Ubc5 and Ubc13 degradation (Shembade *et al*, 2010). Remarkably, mutation of the DUB catalytic site of A20 or its ZF4 also prevents binding of A20 to Ubc5/13 and Ubc5/13 degradation, pointing to a role for ubiquitin-editing as well in these processes (Shembade *et al*, 2010). More recently, a direct non-catalytic mechanism of NF- κ B inhibition by A20 has been proposed, in which simultaneous binding of A20 to long unanchored K63-linked polyubiquitin chains and NEMO is sufficient to block IKK activation by its upstream kinase TAK1 via a still unknown mechanism (Skaug *et al*, 2011). From the above it is clear that A20 can use several DUB-independent mechanisms to regulate NF- κ B signalling. Recent studies found a role for A20 ZF4 and ZF7 in the binding of A20 to K63-linked polyubiquitin chains, which is important for NF- κ B inhibition (Bosanac *et al*, 2010; Skaug *et al*, 2011). However, neither ZF4 nor ZF7 was sufficient for this binding, indicating that multiple ZFs contribute to K63-polyubiquitin binding (Skaug *et al*, 2011). In addition, mutation of either ZF4 or ZF7 impaired the E3 ubiquitin ligase activity of A20 as well as the recruitment of A20 to

NEMO following TNF stimulation (Bosanac *et al*, 2010; Skaug *et al*, 2011), making it difficult to delineate the exact reason of impaired NF- κ B downregulation by the A20 ZF4 and ZF7 mutants.

It has previously been shown that K63 of ubiquitin and the catalytic activity of Ubc13, an E2 that catalyses K63 polyubiquitination, are surprisingly not required for IKK activation by TNF (Xu *et al*, 2009), indicating that A20 may use K63-polyubiquitin independent mechanisms to regulate TNF-induced NF- κ B activation. Therefore, we here investigated whether A20 may also use linear polyubiquitin-dependent mechanisms to downregulate TNF-induced NF- κ B signalling. We show that A20 negatively regulates LUBAC-induced NF- κ B activation and prevents the TNF-inducible interaction between NEMO and LUBAC. Furthermore, we found that inhibition of LUBAC-induced NF- κ B activation by A20 depends on its ZF7 domain, which we here identify as a novel linear ubiquitin-binding domain (LUBID).

Results

A20 is rapidly recruited to LUBAC upon TNF stimulation and inhibits LUBAC-mediated NF- κ B activation

In TNF-stimulated cells, LUBAC activates the NF- κ B pathway by binding to NEMO and conjugating linear polyubiquitin chains onto specific Lys residues in NEMO (Haas *et al*, 2009; Tokunaga *et al*, 2009). To investigate whether A20 might interfere with linear polyubiquitin-mediated NF- κ B signalling, we first analysed whether A20 could be recruited to LUBAC and NEMO upon TNF stimulation. HEK293T cells that constitutively express A20 were stimulated with TNF for different time points and cell lysates were subjected to immunoprecipitation of NEMO or Sharpin, one of the essential components of the LUBAC complex, followed by western blotting for A20, NEMO and the different LUBAC components. TNF-induced I κ B α phosphorylation and degradation served as a control for IKK activation. As expected, TNF induced the rapid recruitment of all three LUBAC components to NEMO (Figure 1A and B), which were present as a preformed complex (Figure 1B). Most importantly, A20 could be co-immunoprecipitated with NEMO as well as Sharpin from cells that were stimulated with TNF for 5 or 15 min (Figure 1A and B), illustrating the TNF-inducible formation of a complex consisting of A20, NEMO and LUBAC. Also, a slightly slower migrating A20 band could be detected upon immunoblotting of Sharpin immunoprecipitates, most likely reflecting the association of Sharpin with a phosphorylated form of A20. Also, Jurkat cells constitutively expressing A20 showed increased binding of A20 to LUBAC and NEMO upon TNF stimulation (Figure 1C and D). In this case, however, significant amounts of A20 could already be co-immunoprecipitated with NEMO and LUBAC from unstimulated cells. It has previously been shown that NEMO and LUBAC are recruited to the TNF-R signalling complex upon TNF stimulation (Haas *et al*, 2009). We therefore investigated the presence of A20 in the TNF-R signalling complex isolated from Jurkat cells that were stimulated with Flag-tagged TNF for different time points. Anti-Flag immunoprecipitation showed the recruitment of A20 along with NEMO and all three LUBAC components to the TNF-R after 5 min TNF stimulation (Figure 2). Interestingly, A20 recruitment still further increased after

Figure 1 TNF induces the recruitment of A20 to NEMO and LUBAC. HEK293T (A, B) or Jurkat (C, D) cells were stimulated with TNF for 5 or 15 min as indicated. Cell extracts were subjected to immunoprecipitation (IP) with anti-NEMO (A, C) or anti-Sharpin (B, D) and co-immunoprecipitated A20, HOIL-1, HOIP and Sharpin were revealed by western blotting with anti-A20, anti-HOIL-1, anti-HOIP and anti-Sharpin, respectively (*=non-specific band; – Ab=IP control setup without antibody). Total expression of A20, NEMO, Sharpin, HOIL-1 and HOIP was analysed by immunoblotting of total cell lysates (TL). NF- κ B activation was evaluated by western blotting for phospho-I κ B α and I κ B α . Results are representative for three independent experiments. Figure source data can be found with the Supplementary data.

15 min TNF stimulation, whereas binding of NEMO and LUBAC already declined.

Next, we further investigated whether the recruitment of A20 into the LUBAC/NEMO signalling complex is associated with the inhibition of LUBAC-mediated NF- κ B activation by A20. We therefore investigated the effect of A20 overexpression on TNF-induced and LUBAC-mediated NF- κ B activation in an NF- κ B reporter gene assay in HEK293T cells. LUBAC-mediated NF- κ B activation was studied by analysing reporter gene expression in response to overexpression of a combination of all three LUBAC components, HOIL-1, HOIP and Sharpin, which was previously shown to activate NF- κ B (Ikeda *et al*, 2011). Co-expression of A20 was found to completely prevent TNF-induced as well as LUBAC-induced

Figure 2 TNF induces the recruitment of A20, LUBAC and NEMO in the TNF-R complex. Jurkat cells were stimulated with Flag-TNF for 5, 15 or 30 min as indicated. Cell extracts were subjected to immunoprecipitation (IP) with anti-Flag to immunoprecipitate the TNF-R complex. Co-immunoprecipitating A20, HOIL-1, HOIP, Sharpin and NEMO were revealed by western blotting with anti-A20, anti-HOIL-1, anti-HOIP, anti-Sharpin and anti-NEMO, respectively. Total expression of A20, HOIL-1, HOIP, Sharpin and NEMO was analysed by immunoblotting of total cell lysates (TL). The band for HOIL-1 that is visible in anti-Flag immunoprecipitates from unstimulated cells represents non-specific binding of HOIL-1 to the immunoprecipitating beads. NF- κ B activation was evaluated by western blotting for phospho-I κ B α and I κ B α . Results are representative for two independent experiments. Figure source data can be found with the Supplementary data.

NF- κ B reporter gene expression (Figure 3). Together with our observation that A20 is recruited into the LUBAC/NEMO signalling complex, these data indicate a potential role for A20 in the negative regulation of LUBAC-mediated signalling to NF- κ B.

A20-ZF7 plays a crucial role in the inhibition of LUBAC-mediated NF- κ B activation

ZF4 and ZF7 of A20 have previously been reported to be important for the inhibitory effect of A20 on TNF-induced NF- κ B activation by interfering with RIP1 and TAK1 mediated IKK activation (Bosanac *et al*, 2010; Skaug *et al*, 2011). To analyse whether these ZFs are also involved in the inhibition of LUBAC-induced NF- κ B activation by A20 as described above, we compared the ability of wild-type A20 and A20-ZF4 (C624A–C627A; ZF4*), A20-ZF7 (C775A–C779A; ZF7*), and A20-ZF4/ZF7 (C624A–C627A/C775A–C779A; ZF4/7*) mutants to inhibit NF- κ B activation induced by TNF stimulation or LUBAC overexpression. Expression levels of A20 and the different mutants are shown in Supplementary Figure 1. Mutation of ZF4 or ZF7 alone did not or only slightly reduce the inhibitory activity of A20 on TNF-induced NF- κ B activation, whereas A20's NF- κ B inhibitory capacity was almost completely disrupted upon mutation of both ZF4 and ZF7 (Figure 3A). When NF- κ B was activated by LUBAC over-

Figure 3 A20 inhibits TNF- and LUBAC-induced NF-κB activation via its ZF7. (A) HEK293T cells were transiently transfected with an NF-κB reporter plasmid, an expression plasmid for β-galactosidase, and an expression plasmid for wild-type A20 (WT) or the indicated A20-ZF4 (C624A–C627A; ZF4*), A20-ZF7 (C775A–C779A; ZF7*), and A20-ZF4/ZF7 (C624A–C627A/C775A–C779A; ZF4/7*) mutants. After 24 h, cells were left untreated or stimulated for 6 h with TNF. Cell lysates were then analysed for luciferase (luc) and β-galactosidase (gal) activity and values are plotted as luc/gal to normalize for possible differences in transfection efficiency. (B) HEK293T cells were transiently transfected as in (A), but cotransfected with expression plasmids for all three LUBAC components (HOIL-1, HOIP, Sharpin) to activate NF-κB. After 24 h, cell lysates were made and analysed as in (A). Values are the mean of triplicates ± s.d. Results are representative for three independent experiments.

expression, however, mutation of ZF7 only was sufficient to completely prevent the inhibitory effect of A20 (Figure 3B), indicating a crucial role for ZF7 in the negative regulation of LUBAC-mediated NF-κB activation by A20. We next investigated whether expression of A20-ZF7 (aa 758–790) alone (ZF7_{only}) was able to prevent TNF- and LUBAC-induced NF-κB activation. Because of poor expression of the A20-ZF7 polypeptide and to facilitate its detection by western blotting, A20-ZF7 was fused to GFP. As a negative control, we expressed the corresponding mutant ZF7 (C775A–C779A) fusion protein (ZF7*_{only}). Expression levels of all fusion proteins are shown in Supplementary Figure 1. Overexpression of ZF7_{only} significantly reduced NF-κB activation in response to both TNF and LUBAC overexpression, which was completely disrupted upon mutation of the ZF7 structure (Figure 4). Interestingly, whereas full-length A20 was more potent than ZF7_{only} to inhibit TNF-induced NF-κB activation,

Figure 4 A20-ZF7_{only} inhibits TNF and LUBAC-induced NF-κB activation. (A) HEK293T cells were transiently transfected with an NF-κB reporter plasmid, an expression plasmid for β-galactosidase, and an expression plasmid for wild-type A20 (WT), A20-ZF7_{only} (ZF7_{only}) or the corresponding mutant A20-ZF7_{only} (C775A–C779A) (ZF7*_{only}). After 24 h, cells were left untreated or stimulated for 6 h with TNF. Cell lysates were then analysed for luciferase (luc) and β-galactosidase (gal) activity and values are plotted as luc/gal to normalize for possible differences in transfection efficiency. (B) HEK293T cells were transiently transfected as in (A), but cotransfected with expression plasmids for all three LUBAC components (HOIL-1, HOIP, Sharpin) to activate NF-κB. After 24 h, cell lysates were made and analysed as in (A). Values are the mean of triplicates ± s.d. Results are representative for three independent experiments.

both were equally effective when NF-κB was activated by LUBAC overexpression (compare Figure 4A and B). The latter observation together with our finding that mutation of ZF7 in full-length A20 has much more drastic effects on NF-κB activation induced by LUBAC compared with TNF, suggests a linear ubiquitin-dependent function for ZF7 rather than a dependency on other ubiquitin chain types such as K48- or K63-linked polyubiquitin. In contrast, the latter chains may be more important for A20's ability to inhibit TNF-induced NF-κB activation via its ZF4 (Bosanac *et al*, 2010; Skaug *et al*, 2011).

Our finding that expression of ZF7_{only} as such is sufficient to inhibit TNF-induced NF-κB activation is quite remarkable. As its effect was demonstrated in an NF-κB-dependent reporter gene assay upon overexpression in HEK293T cells that also express endogenous A20, one could argue that the effect of ZF7_{only} may be dependent on endogenous A20

or be limited to an artificial reporter gene setup. We therefore compared the TNF-induced expression of iNOS, a NF- κ B-dependent gene, in A20-deficient MEF cells reconstituted with either ZF7_{only}, ZF7*_{only} or full-length A20. Similar to our findings in HEK293T cells, overexpression of full-length A20 as well as ZF7_{only} in A20-deficient MEF cells inhibited TNF-induced iNOS expression (Figure 5). In addition, we were unable to co-immunoprecipitate endogenous A20 with ZF7_{only} from HEK293T cells (see below; Figure 7A). These data therefore exclude a role for endogenous A20 in the NF- κ B inhibitory activity of ZF7_{only}.

A20 and A20-ZF7 bind linear polyubiquitin

To further characterize the linear ubiquitin-dependent function of ZF7, we considered its ubiquitin-binding potential. Previous studies showed a role for ZF4 and ZF7 in the binding of A20 to K63-linked polyubiquitin chains (Bosanac *et al*, 2010; Skaug *et al*, 2011). However, neither ZF4 nor ZF7 was sufficient for this binding (Skaug *et al*, 2011). As these studies did not investigate the potential binding to other types of polyubiquitin, we therefore re-examined the ability of ZF7 to bind K48-linked, K63-linked, and linear polyubiquitin chains *in vitro*. A GST pull-down assay with recombinant purified GST fusion proteins showed that ZF7_{only} preferentially binds linear polyubiquitin (Figure 6A), similar to the UBA1 domain of ABIN-1, which was previously established as a linear polyubiquitin-binding domain and used here as a positive control (Wagner *et al*, 2008). Disruption of the ZF structure by mutation of C775A–C779A completely abolished the inter-

action of ZF7_{only} with linear polyubiquitin. We also assessed the binding of ZF7_{only} to linear ubiquitin chains in competition with K48- or K63-linked polyubiquitin chains of the same length as described before (Kensche *et al*, 2012). Purified ZF7_{only} was incubated with equal concentrations of different types of polyubiquitin in the same tube and binding of ZF7_{only} was monitored by washing off unbound chains and western blot analysis. Since ubiquitin chains of the same length but with different linkage display different mobility on SDS-polyacrylamide gel electrophoresis, it is possible to distinguish the different chain types by using the same anti-ubiquitin antibody. Incubation of GST-ZF7_{only} with different chain types led to a preferential binding of linear polyubiquitin in competition with either K48- or K63-polyubiquitin (Figure 6B). Taken together, our data identify ZF7 as a specific LUBID.

In vitro incubation of different types of polyubiquitin with recombinant Flag-A20 followed by western blotting and detection with anti-ubiquitin, revealed the potential of full-length A20 to bind linear as well as K48- and K63-linked polyubiquitin chains (Figure 6A). Most likely this reflects a role for other ZFs including ZF4, which was previously shown to have higher affinity for K63-linked tri-ubiquitin than for K48-linked or linear tri-ubiquitin (Bosanac *et al*, 2010).

A20-ZF7 prevents the TNF-induced formation of a NEMO/LUBAC signalling complex

LUBAC conjugates linear polyubiquitin chains on NEMO, RIP1, as well as on the LUBAC components themselves (Gerlach *et al*, 2011; Ikeda *et al*, 2011; Tokunaga *et al*, 2011). Because the UBA1 domain of NEMO as well as the NZF domain of the HOIL-1 and Sharpin subunits of LUBAC have previously been shown to bind linear polyubiquitin chains (Rahighi *et al*, 2009; Sato *et al*, 2011), it is believed that linear ubiquitination of NEMO and Sharpin/HOIL-1 enables the formation of a stable and functional NEMO/LUBAC complex. Our finding that A20 and ZF7_{only} bind linear polyubiquitin chains let us hypothesize that A20 and ZF7_{only} may at least partially inhibit NF- κ B activation by preventing NEMO/LUBAC complex formation via competitive binding to linear polyubiquitin. We therefore first investigated if ZF7_{only}, similar to full-length A20 (Figure 1), can interact with NEMO and LUBAC upon TNF stimulation. Indeed, transfected ZF7_{only} could be co-immunoprecipitated with all three endogenous LUBAC components (Figure 7A) and NEMO (Figure 7B) upon 5 min TNF stimulation of HEK293T cells, indicating that ZF7 is sufficient for the TNF-inducible recruitment of A20 and ZF7_{only} to the NEMO/LUBAC complex. Furthermore, the requirement for TNF stimulation to observe an interaction of ZF7_{only} with LUBAC could be overcome by overexpression of LUBAC (Figure 7C), consistent with the ability of LUBAC overexpression to induce NF- κ B activation and its inhibition by co-expression of ZF7_{only} (Figure 4B). Most interestingly, LUBAC overexpression also drastically increased ZF7_{only} co-immunoprecipitation with NEMO (Figure 7C, lane 4), most likely reflecting the LUBAC-induced linear ubiquitination of NEMO and its recognition by ZF7_{only}. Next, we analysed if the observed binding of A20 or ZF7_{only} to NEMO/LUBAC was able to prevent or disrupt the TNF-induced formation of a NEMO/LUBAC signalling complex. Indeed, co-immunoprecipitation studies showed that overexpression of A20 or ZF7_{only} completely

Figure 5 A20-ZF7_{only} inhibits TNF-induced iNOS expression in reconstituted A20-deficient MEF cells. A20-deficient MEF cells were reconstituted by transfection with expression vectors for either wild-type (WT) A20, A20-ZF7_{only} (ZF7_{only}) or the corresponding mutant A20-ZF7_{only} (C775A–C779A) (ZF7*_{only}). After 24 h, cells were left untreated or stimulated with 10 ng/ml mouse TNF for 6 h. iNOS expression was analysed via Q-PCR. Values are the mean of triplicates $\pm$ s.d. Results are representative for two independent experiments. Expression levels of transfected GFP-A20 fusion proteins were verified via western blotting with anti-GFP. All samples are from the same experiment; ZF7_{only} and ZF7*_{only} samples (non-stimulated and stimulated, each in triplicate) were loaded on the same gel, but WT triplicates were loaded on a separate gel. Gels/blots were processed in parallel and loading controls (anti-actin) are from the same gel/blot and from the same lane as the corresponding GFP-A20 fusion protein. A representative signal is shown at the bottom of the graph. Figure source data can be found with the Supplementary data.

Figure 6 A20-ZF7_{only} preferentially binds linear polyubiquitin in competition with other chain types. **(A)** Recombinant GST fusion proteins of the ABIN-1 UBAN domain, its ubiquitin-binding deficient mutant (UBAN DF/NA), A20-ZF7_{only} and A20-ZF7_{only} (C775A-C779A) (=ZF7*_{only}) immobilized on glutathione-sepharose beads were incubated with linear, K48 or K63 polyubiquitin chains of specific lengths (as indicated between brackets) for 3 h at 4°C. Polyubiquitin binding was detected by GST pull-down (GST-PD) and western blot analysis with anti-ubiquitin. GST fusion proteins were detected by western blot analysis with anti-GST. Binding of different polyubiquitin chain types to full-length A20 (last lane) was analysed using recombinant Flag-A20 immobilized on agarose beads using anti-Flag immunoprecipitation (IP) (* = antibody light chain). Results are representative of three independent experiments. **(B)** GST fusion proteins of A20-ZF7_{only} and A20-ZF7*_{only} (C775A-C779A) (=ZF7*_{only}) were incubated with linear tri-ubiquitin (upper panel) or tetra-ubiquitin (lower panel) in competition with K63-linked tri-ubiquitin and K48-tetra-ubiquitin, respectively. Pull downs and western blotting were performed as in **(A)**. GST fusion proteins were revealed by Ponceau S staining of the blots. Results are representative of three independent experiments. Figure source data can be found with the Supplementary data.

prevents the TNF-induced binding of NEMO with all three LUBAC components (Figure 8; Supplementary Figure 2). Mutation of ZF7, which prevents its linear polyubiquitin-binding potential, also impaired the ability of A20 or ZF7_{only} to disrupt the NEMO-LUBAC interaction. In contrast, mutation of ZF4 (ZF4*) had no effect as it did not affect the ability of A20 to disrupt the NEMO-Sharpin interaction (Supplementary Figure 3). These results support a model in which competitive binding of A20 or ZF7 to linear polyubiquitin is able to prevent the TNF-induced formation of a NEMO/LUBAC complex and eventually TNF-induced NF-κB activation.

Discussion

Signalling cascades controlling NF-κB activation involve interactions among numerous proteins and protein complexes, including a wide variety of ubiquitin chains. Recent evidence indicates that LUBAC and its linear ubiquitin chain generating activity are required for the full activation of TNF-induced NF-κB signalling (Haas *et al*, 2009; Tokunaga *et al*, 2009, 2011; Gerlach *et al*, 2011; Ikeda *et al*, 2011; Verhelst *et al*, 2011). Our results illustrate a novel role for A20 in the negative regulation of LUBAC-mediated NF-κB activation and suggest that A20 attenuates NF-κB signalling, at least in part, by binding to linear polyubiquitin chains. We started this study with the observation that A20 rapidly binds NEMO and LUBAC upon TNF stimulation, and is recruited together

with NEMO and LUBAC into the TNF-R signalling complex. Binding of A20 to NEMO was initially reported by Zhang *et al* (2000), but the underlying molecular mechanism and its biological relevance were not defined. We now further demonstrate a key role for ZF7 in the TNF-induced binding of A20 to the NEMO/LUBAC signalling complex. Importantly, we identified ZF7 as a novel LUBID. TNF stimulation induces the LUBAC-mediated linear ubiquitination of NEMO and the different LUBAC components in the TNF-R complex. Besides being covalently modified by linear chains, NEMO, HOIL-1 and Sharpin also non-covalently bind linear polyubiquitin via their UBAN and NZF domains, respectively, which is believed to stabilize the TNF-R signalling complex and enhance TNF-induced NF-κB activation (Haas *et al*, 2009). Our finding that ZF7 binds linear polyubiquitin suggests that linear ubiquitination of NEMO/LUBAC also mediates the recruitment of A20 into the TNF-R/NEMO/LUBAC signalling complex. We further showed that A20 overexpression disrupts the TNF-inducible binding of NEMO to all three LUBAC components in a ZF7-dependent manner, supporting a model in which A20 inhibits TNF-induced and LUBAC-mediated NF-κB activation via competitive binding to linear polyubiquitin chains conjugated to NEMO, LUBAC, RIP1 and possibly other TNF-R signalling components. Recently, Skaug *et al* (2011) showed that long unanchored K63-linked polyubiquitin chains can also mediate the recruitment of A20 to NEMO, which is partially dependent on ZF7. We can therefore not exclude a similar role for A20 binding to

Figure 7 TNF stimulation or overexpression of LUBAC induces the binding of A20-ZF7_{only} to NEMO and LUBAC components. (A) HEK293T cells were transiently transfected with GFP-A20-ZF7_{only} and 24 h later stimulated with TNF for the indicated times. Cell extracts were subjected to anti-GFP immunoprecipitation and co-immunoprecipitating HOIL-1, HOIP, Sharpin and A20 were detected by western blotting. Total cell lysates (TL) were analysed for the expressed proteins using western blotting. NF-κB activation was evaluated by western blotting for phospho-IκBα and IκBα. Results are representative of three independent experiments. (B) HEK293T cells were transiently transfected with GFP-A20-ZF7_{only} and 24 h later stimulated with TNF for the indicated times. Cell extracts were subjected to anti-NEMO immunoprecipitation and co-immunoprecipitating GFP-A20-ZF7_{only} was detected by western blotting with anti-GFP (* = antibody heavy chain). Total cell lysates (TL) were analysed for the expressed proteins using western blotting. Results are representative of three independent experiments. (C) HEK293T cells were transiently transfected with GFP-A20-ZF7_{only}, Flag-NEMO, or a combination (indicated as His-LUBAC) of His-Sharpin, His-HOIL-1, His-HOIP, as indicated. The day after, cell extracts were subjected to anti-GFP immunoprecipitation (IP) and co-immunoprecipitating LUBAC and NEMO proteins were detected by western blotting with anti-His or anti-Flag, respectively (* = non-specific band). Total cell lysates (TL) were analysed for the expressed proteins using western blotting. Results are representative of three independent experiments. Figure source data can be found with the Supplementary data.

unanchored linear polyubiquitin chains in its recruitment to NEMO and the prevention of NEMO/LUBAC complex formation. As the N-terminal domain of A20 has DUB activity, one could speculate that A20 may also prevent NEMO/LUBAC complex formation by removing linear ubiquitin chains from NEMO or LUBAC components in a ZF7-dependent manner. However, the latter possibility is unlikely as recombinant A20 was previously shown to specifically deubiquitinate K11- and K48-linked chains, but not linear polyubiquitin (Komander and Barford, 2008; Bosanac *et al*, 2010). Similarly, we were unable to detect any DUB activity on linear polyubiquitin with A20 immunoprecipitated from transfected cells (Supplementary Figure 4). Moreover, the fact that NEMO/LUBAC complex formation can already be disrupted by ZF7_{only} which lacks any DUB activity excludes a role for A20-mediated removal of linear ubiquitin from NEMO or LUBAC.

Previous studies have also implicated an important role for ZF4 in the biological activity of A20. More specifically, ZF4

was originally shown to be involved in A20-mediated conjugation of K48-linked polyubiquitin chains to RIP1, leading to its proteasomal degradation (Wertz *et al*, 2004). More recently, ZF4 was shown to bind mono-ubiquitin and K63-linked polyubiquitin, and to have much lower affinity for K48-linked or linear polyubiquitin (Bosanac *et al*, 2010). The ability of ZF4 and ZF7 to bind different types of polyubiquitin chains may also reflect our observation that complete disruption of A20's inhibitory activity on TNF-induced NF-κB activation requires mutation of both ZF4 and ZF7, whereas mutation of ZF7 is sufficient to disrupt A20's inhibitory activity on NF-κB activation induced by LUBAC overexpression. Specific binding of linear chains by ZF7 may allow A20 to affect linear ubiquitin-mediated NEMO/LUBAC binding, whereas binding of ZF4 to K63-polyubiquitin may also allow the recognition by A20 of upstream K63-ubiquitinated signalling molecules such as RIP1 and TRAF2.

The UBAN (coiled-coil) domain that is present in NEMO, optineurin and ABINs, and the NZF (zinc-finger) domain that

Figure 8 A20 and A20-ZF7_{only} prevent TNF-induced binding of NEMO to Sharpin and HOIP. HEK293T cells were transiently transfected with E-tagged A20 (E-A20), E-tagged A20 (C775A–C779A) (E-A20-ZF7*), GFP-A20, GFP-A20-ZF7_{only} (GFP-ZF7), or GFP-A20-ZF7_{only} (C775A–C779A) (GFP-ZF7*). After 24 h, cell extracts were subjected to immunoprecipitation (IP) with anti-NEMO and co-immunoprecipitating Sharpin and HOIP was detected by immunoblotting with anti-Sharpin or anti-HOIP, respectively. Total expression of A20, NEMO, Sharpin or HOIP was analysed by immunoblotting of total cell lysates (TL) with anti-E, anti-GFP, anti-NEMO, anti-Sharpin or anti-HOIP, respectively. Results are representative of three independent experiments. Figure source data can be found with the Supplementary data.

is present in the LUBAC components HOIL-1 and Sharpin, are the only other ubiquitin-binding domains known to specifically bind linear chains. To elucidate the mechanism of linear polyubiquitin binding by A20–ZF7, it will be interesting to solve the crystal structure of A20–ZF7 in complex with linear di-ubiquitin and to do a structural comparison with the binding of linear di-ubiquitin by the UBAN and NZF domains of NEMO and HOIL-1, respectively. It was already shown that the basis for the binding to both proximal and distal ubiquitin moieties is completely different between NEMO UBAN and HOIL-1 NZF (Rahighi *et al*, 2009; Sato *et al*, 2011). However, both the NZF of HOIL-1 and UBAN of NEMO appear to be unique in not binding the canonical Ile44-centred hydrophobic patch of the proximal Ub and instead recognizing the Phe4-centred patch, suggesting that the Phe4-centred patch may be a common property of LUBIDs. It should be mentioned that in a cellular context, the specificity of polyubiquitin binding may be further determined by A20 dimerization, posttranslational modifications, or A20-binding proteins, including ABINs and TAX1BP1 (Verstrepen *et al*, 2009, 2011), that themselves also bind different types of ubiquitin chains (Iha *et al*, 2008; Wagner *et al*, 2008).

Our finding that ZF7 binds linear polyubiquitin chains supports a model in which A20 prevents the binding of NEMO to LUBAC via competitive binding to linear polyubiquitin chains. However, we do not exclude that other mechanisms also contribute to the ZF7 dependency of A20's NF-κB inhibitory function. Recently, the ZF region of A20 was shown to prevent NF-κB activation by disrupting E2 and E3

enzyme complexes via competitive binding (Shembade *et al*, 2010). Since the region of A20 comprising ZF5 till ZF7 has been shown to contribute to the specific binding of A20 to Ubch5a (the E2 enzyme that together with LUBAC mediates linear ubiquitination), one could speculate that A20 similarly prevents the formation of a functional Ubch5a–LUBAC complex via competitive binding to Ubch5a. Also, the C-terminal ZFs of A20 have been implicated in the targeting of A20 to a lysosome-associated endocytic membrane compartment and the lysosomal degradation of TRAF2 (Li *et al*, 2008, 2009). The above-mentioned studies mainly rely on the use of C-terminal deletion mutants of A20 in which multiple ZFs were deleted and it will be interesting to analyse in more detail the specific role of ZF7 using site-specific mutagenesis of ZF7. It is clear that A20 can use multiple mechanisms to interfere with TNF-induced NF-κB activation and their relative contribution may be dependent on multiple factors such as A20 expression levels, posttranslational modifications or binding of A20 to other proteins. In addition, different mechanisms may be active at different time points and in different cell types. In this context, it is worth mentioning that our findings were obtained in cells that constitutively express A20, such as lymphocytes, allowing the rapid recruitment of A20 along with NEMO and LUBAC in the TNF-R signalling complex. Many other cell types, however, only express significant A20 levels several hours after stimulation with TNF or other pro-inflammatory stimuli, and the role of LUBAC-mediated linear ubiquitination and its regulation by A20 at these late time points remain to be studied.

Polymorphisms of A20 are associated with multiple autoimmune and inflammatory diseases, including rheumatoid arthritis, systemic lupus erythematosus and Crohn's disease, and mutations of A20 result in multiple subsets of B cell lymphomas and Sézary syndrome (Vereecke *et al*, 2009). Together with the autoimmune phenotype of several tissue-specific A20 knockout mice (Tavares *et al*, 2010; Chu *et al*, 2011; Hammer *et al*, 2011; Hovelmeyer *et al*, 2011; Kool *et al*, 2011; Matmati *et al*, 2011), these data indicate that A20 dysfunction may be implicated in disease pathogenesis. Importantly, A20 mutants lacking ZF7 and missense mutations in A20 have been found in B cell lymphoma (Kato *et al*, 2009; Schmitz *et al*, 2009), highlighting the physiological significance of linear polyubiquitin binding by ZF7 in NF-κB suppression. Final proof of the specific role of ZF7 in mammalian physiology will likely require the generation of a knockin mouse lacking a functional ZF7. Furthermore, our data do not only provide a molecular explanation for the observed association of ZF7 mutations with B cell lymphoma, they also suggest that ZF7 polypeptides or peptidomimetics may be promising therapeutic agents for B cell lymphoma and autoimmune diseases.

Materials and methods

Cells and reagents

HEK293T cells (human embryonic kidney cells expressing SV40 large T antigen) were a gift from Dr M Hall (Department of Biochemistry, University of Birmingham, UK). The cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM L-Glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.4 mM sodium pyruvate. Jurkat-E (human leukaemic T lymphoma cell line) cells were grown in

RPMI1640 medium, supplemented with the same reagents as mentioned above. A20-deficient MEF cells (kind gift of Dr Averil Ma, UCSF, CA) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, 2 mM L-Glutamine, 100 IU/ml penicillin and 0.1 mg/ml streptomycin. Recombinant human TNF and Flag-TNF were expressed in *Escherichia coli* in our laboratory and purified to at least 99% homogeneity. TNF had a specific biological activity of 2.58×10^8 IU/mg purified protein, as determined with the International Standard (code 87/650) (National Institute for Biological Standards and Control, Potters Bar, UK). Protein G-Sepharose was from Sanbio B.V. (Uden, The Netherlands). The following antibodies were used: anti-E polyclonal antibody (Abcam, Cambridge, UK), anti-actin monoclonal antibody (MP Biomedicals, Illkirch Cedex, France), anti-FLAG M2 monoclonal antibody (Sigma, St Louis, MO, USA), anti-GFP monoclonal antibody (Clontech Saint-Germain-en-Laye, France), anti-ubiquitin monoclonal antibody (P4D1; Eurogentec, Seraing, Belgium), anti-GST (clone 27457701V, Amersham, Diegem, Belgium), anti-His monoclonal antibody (R933-25, Invitrogen, NY, USA), anti-NEMO polyclonal antibody (clone FL-419, Santa Cruz, Heidelberg, Germany), anti-NEMO monoclonal antibody (clone 68351A, Pharmingen, Erembodegem, Belgium), anti-Sharpin polyclonal antibody (ab69507, Abcam, Cambridge, UK or SAB1408056, Sigma, St Louis, MO, USA), anti-HOIL-1 (sc365523, Santa Cruz, Heidelberg, Germany), anti-HOIP (ab85294, Abcam, Cambridge, UK), anti-A20 monoclonal antibody (60-6629-82, eBioscience, San Diego, CA, USA). A secondary mouse or rabbit antibody conjugated with HRP was obtained from Amersham (Diegem, Belgium), or conjugated with Dylight 680 or 800 from Pierce (Rockford, USA). Recombinant K48-linked and K63-linked polyubiquitin chains (Ub3-7) were purchased from Boston Biochem (Cambridge, MA, USA); linear polyubiquitin chains (Ub7) from PBL Biomedical Laboratories (NJ, USA). Tetra-K48- and tri-K63-linked ubiquitin chains were produced as described earlier (Komander *et al*, 2008). Linear tri- and tetra-ubiquitin were expressed as GST fusion proteins in *E. coli* BL21, purified, and cleaved with thrombin protease (Biotinylated Thrombin Kit, Novagen) according to the manufacturer's instructions. Thrombin buffer was exchanged with 50 mM Tris-HCl pH 7.5.

Expression plasmids

pNFconluc (LMBP3248), which contains the luciferase gene under the control of a minimal promoter, preceded by three NF- κ B sites, was a gift from Dr A Israël (Institut Pasteur, Paris, France). pACT β gal (LMBP4341), containing the β -galactosidase gene after the β -actin promoter, was from Dr J Inoue (Institute of Medical Sciences, Tokyo, Japan). pcDNA3-HOIL-1-V5/His, pcDNA3-HOIP-V5/His and pcDNA3-Sharpin-V5/His were from Dr H Walczak (Tumour Immunology Unit, Faculty of Medicine Imperial College, London, UK). pGEX-GST-UBAN and pGEX-GST-UBAN DF/NA were described previously (Wagner *et al*, 2008). pCD-FLAG-NEMO, pCAGGS-E-A20 (LMBP 3778), pCAGGS-E-A20 (C624A-C627A) (ZF4*; LMBP 6563), pCAGGS-E-A20 (C775A-C779A) (ZF7*; LMBP 6569), and pCAGGS-E-A20 (C624A-C627A/C775A-C779A) (ZF4/*; LMBP 6570) were constructed by PCR mutagenesis. Plasmids indicated with an accession number (LMBP xxxx) are available from the plasmid collection of the Belgian Coordinated Collections of Micro-organisms (BCCM/LMBP; Department of Biomedical Molecular Biology, Ghent University, Belgium; <http://bccm.belspo.be/index.php>). pCAGGS-GFP-A20 was generated by PCR using the following forward (5'-GCTCTAGAGCCTCTGCTA ACC-3') and reverse (5'-TTTCTCTTTGCGGCCGCTTGTATAGT TCATCCATGCC-3') primers, creating a restriction site at the 5' end for *Xba*I and for *Not*I at the 3' end. The PCR product was cloned *Xba*I-*Not*I in pCAGGS-E-A20. pCAGGS-GFP-A20-ZF7 (LMBP 6066; A20-ZF7_{only}) was generated by PCR using the following forward (5'-CCGCTCAGAGCTGGTGGTGGTGGTGGTAAGCAGC TTGCCGGGCCCC-3') and reverse (5'-CCGCTCAGAGTATGCCATACAT CTGCTTG-3') primers, creating a restriction site at the 5' and 3' end for *Xho*I. Afterwards the PCR product was cloned in the *Xho*I site of pCAGGS-E-A20. pCAGGS-GFP-A20-ZF7 (C775A-C779A) (LMBP 6117; ZF7_{only}) was constructed by PCR mutagenesis.

NF- κ B-dependent reporter assays

HEK293T cells were seeded at 4×10^4 cells/well in 24-well plates. Cells were transiently transfected the next day by DNA calcium

phosphate coprecipitation. Each transfection contained 20 ng of pNFconluc, 20 ng pACT β gal and 20 ng of a specific A20 expression plasmid. The total amount of DNA per well was kept constant at 200 ng by adding empty pCAGGS vector. After 24 h, cells were stimulated with 1000 IU/ml human TNF or left untreated. After 6 h, cells were lysed in luciferase lysis buffer, followed by luc and β -gal assays as described (Verstrepen *et al*, 2008).

Co-immunoprecipitation and western blot analysis

HEK293T cells were seeded at 1.2×10^6 cells on 90-mm petri dishes and transfected with 5 μ g DNA. The day after, cells were lysed in E1A buffer (50 mM Hepes pH 7.6, 250 mM NaCl, 5 mM EDTA and 0.5% NP-40) supplemented with protease inhibitors (2.1 μ M leupeptine, 0.15 μ M aprotinin and 1 mM pepstatin) and phosphatase inhibitors (200 μ M sodium orthovanadate, 10 mM sodium fluoride and 5 μ g/ml β -glycerophosphate). Interactions of endogenously expressed proteins were investigated in LUBAC lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl and 1% Triton X-100). Cell extracts were centrifuged at 14 000 r.p.m. for 15 min and nine-tenths of the soluble fraction was incubated with 1 μ g antibodies for 1 h at 4°C and then mixed with protein G-sepharose beads. Incubation was continued for 2 h. The beads were washed four times with 1 ml lysis buffer and immunoprecipitates were separated by SDS-PAGE and immunoblotted with specific antibodies as indicated. The remainder of each cell lysate was used for detection of total protein expression by western blotting.

For TNF-R complex analysis, 5×10^7 Jurkat-E cells were stimulated with 2.5 μ g Flag-TNF followed by cell lysis in GST lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.5 mM DTT, 1% Triton X-100, 10 mM N-ethylmaleimide, 10 μ M ZnCl₂) supplemented with protease and phosphatase inhibitors. Cell extracts were then further sonicated for complete lysis. At that time point, Flag-TNF was added to extracts prepared from non-stimulated cells. After centrifugation at 14 000 r.p.m. for 15 min, the TNF-R complex was isolated via immunoprecipitation using anti-Flag-coupled agarose beads (Sigma, St Louis, MO, USA) during 3 h at 4°C. The beads were washed three times with 0.5 ml lysis buffer and immunoprecipitates were separated by SDS-PAGE and immunoblotted with specific antibodies as indicated. The remainder of each cell lysate was used for detection of total protein expression by western blotting.

Ubiquitin-binding assay

Recombinant GST fusion proteins immobilized on glutathione-sepharose beads or 100 ng recombinant Flag-A20 (BPS Bioscience, Gentaur, The Netherlands) immobilized on anti-Flag-coupled agarose beads were incubated with 1 μ g K48-linked, K63-linked or linear polyubiquitin chains for 18 h at 4°C in pull-down buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 μ M ZnCl₂, 0.5 mM DTT). The beads were washed three times with pull-down buffer and proteins were eluted with SDS-sample buffer and analysed by SDS-PAGE and immunoblotting with anti-ubiquitin.

Transfection of MEF cells, RNA isolation and Q-PCR

A20-deficient MEF cells were transfected at 2×10^6 cells/setup using the ME2 nucleofactor kit (Amaxa, Lonza Verviers Sprl) with 5 μ g of the following plasmids: empty vector pCAGGS (LMBP 2453), pCAGGS-GFP-A20-ZF7 (LMBP 6066), pCAGGS-GFP-A20-ZF7 (C775A-C779A) (LMBP 6117) or pCAGGS-GFP-A20 (LMBP 6406). After electroporation, cells were seeded at 200 000 cells/six-well. After 24 h, cells were stimulated with 10 ng/ml mouse TNF for 6 h or left untreated. For RNA isolation, cells were lysed in 500 μ l Trizol (Invitrogen) and 100 μ l chloroform (Merck, VWR International) was added, followed by thorough mixing. Samples were centrifuged for 5 min at 14 000 r.p.m. and the upper phase was transferred to a new eppendorf tube. Subsequently, an equal amount of 70% EtOH was added and samples were transferred to a spin column. RNA isolation was further done using the Aurum total RNA mini kit (Bio-Rad) according to the manufacturer's protocol. RNA was eluted using 40 μ l of the included elution buffer. RNA concentration was measured and 0.5 μ g of RNA was used to make cDNA using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. In all, 10.5 ng cDNA was used for every Q-PCR reaction with the LC 480 Sybr Green I master kit (Roche). RPL13A and HPRT1 were used as house keeping genes to normalize gene

expression. The following Q-PCR primers were used: iNOS (forward: CAGCTGGGCTGTACAAACCTT; reverse: CATTTGGAAGTGAAGCGTTTCG), RPL13A (forward: CCTGCTGCTCTCAAGGTT; reverse: TGGTTGTCACTGCCTGGTACTT), HPRT (forward: AGTGTGGATACAGGCCAGAC; reverse: CGTGATTCAAATCCTGAAGT). For protein isolation, cells were washed once with ice-cold PBS and lysed in E1A buffer supplemented with protease and phosphatase inhibitors.

Supplementary data

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

Acknowledgements

We thank Dr H Walczak, Dr A Israel, Dr J Inoue and Dr M Hall for the generous gift of reagents. Research in the authors laboratory

was supported by grants from the 'Interuniversity Attraction Poles' (IAP6/18 and IAP7), the 'Fund for Scientific Research—Flanders' (FWO; Grants G.0619.10, G.0089.10, 3G023611, 1509712N), the 'Belgian Foundation Against Cancer', the 'Strategic Basic Research Programme' of the 'Instituut voor Innovatie door Wetenschap en Technologie' (IWT), the 'Queen Elisabeth Medical Foundation', and the 'Hercules', 'Concerted Research Actions' (GOA), and 'Group-ID MRP' initiatives of Ghent University. KV was supported as a predoctoral fellow from the IWT and LV is a postdoctoral fellow of the FWO.

Author contributions: KV, MK, LM, LV, TK and IC designed and performed the experiments. KV, IC and RB wrote the manuscript. IC, ID and RB supervised the work.

Conflict of interest

The authors declare that they have no conflict of interest.

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