Involvement of A20 in the molecular switch that activates the non-canonical NF-κB pathway

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The non-canonical NF-κB pathway is crucial for the immune system. A critical event in activation of the non-canonical pathway is the attenuation of NF-κB-inducing kinase (NIK) degradation, which is promoted by continuous polyubiquitination of NIK catalyzed by the NIK ubiquitin-ligase complex composed of cellular inhibitor of apoptosis protein 1 and 2 (cIAP1/2), TNF receptor-associated factor 2 (TRAF2), and TRAF3. However, the molecular mechanism of stimulation-dependent NIK stabilization remains poorly understood. Here, we show that A20, a ubiquitin-editing enzyme, promotes efficient activation of the non-canonical pathway independent of its catalytic activity. A20 directly binds to cIAP1 through the seventh zinc finger of A20, resulting in dissociation of the TRAF2/TRAF3 interaction, thereby inactivating the ligase complex to stabilize NIK. Given that A20 negatively regulates the canonical pathway, A20 is likely involved in the molecular switch that promotes the transition from canonical to non-canonical activation for proper control of the immune system.

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addition to TAK1 activation, IKK activation requires the stimulation-induced conjugation of linear polyubiquitin chains to NEMO catalyzed by the linear ubiquitin chain assembly complex (LUBAC), which may induce oligomer formation or a conformational change in NEMO to activate the IKK complex^{20–24}. In the non-canonical pathway, NF-kB-inducing kinase (NIK) phosphorylates and activates IKK\(\alpha\) homodimer, which phosphorylates p100 to promote p100 processing to p52. Under unstimulated conditions, NIK is persistently degraded by the proteasome due to K48 polyubiquitination by cIAP1/2, the TRAF2/TRAF3 heterodimer acts as a molecular bridge between NIK and cIAP1/2 (Ref. 3, 4). Stimulation that activates the non-canonical pathway induces stabilization and activation of NIK, occasionally with concomitant degradation of TRAF2 and TRAF3 (Ref. 15, 16). Although the molecular switch that attenuates NIK degradation is a critical component in understanding the non-canonical pathway, its mechanism remains to be elucidated.

A20 is a ubiquitin-editing enzyme that potently suppresses the canonical pathway. The N-terminal ovarian tumor (OTU) domain of A20 can deubiquitate K63 polyubiquitin of RIP1, and the C-terminal zinc-finger (ZnF) region subsequently acts as an E3 ligase to add the K48 polyubiquitin chain to RIP1, thereby promoting its proteasomal degradation^{25}. A20 also inactivates the E2/E3 ubiquitination complexes required for activation of the canonical pathway by inhibiting the association of UBC13 with TRAF6, TRAF2 with cIAP1/2, or UBC5C with TRAF6, and subsequently promoting proteasomal degradation of E2 enzymes^{26}. Moreover, A20 impairs IKK\(\beta\) activation by binding to a K63 linear polyubiquitin chain without requiring the deubiquitase and ubiquitin ligase activities of A20 (Ref. 20–22). These previous studies clarified the inhibitory roles of A20 in the canonical pathways. However, the roles of A20 in the non-canonical pathway are largely unknown. In this paper, we show, for the first time, that A20 functions as a positive regulator of the non-canonical pathway by promoting the stimulation-dependent stabilization of NIK.

**Results**

**A20 is crucial for the efficient activation of the non-canonical NF-\(\kappa\)B pathway.** To explore novel roles of A20, we first generated human HEK293T cells expressing A20 N-terminally fused to a tandem affinity purification (TAP)–tag consisting of FLAG and double strep tags (TAP-A20-overexpressing HEK293T cells) by retrovirus-mediated gene transfer. A20-containing complexes were then isolated from the cells and subjected to mass spectrometry analysis. cIAP1 and TRAF2 are found to be components of the A20-containing complex, which led us to think that A20 might be involved in the non-canonical NF-kB pathway. To analyze the involvement of A20 in the non-canonical pathway, the human breast cancer cell line MDA-MB-231 was transfected with control small interfering RNA (siRNA) or two distinct siRNAs for A20 (A20-1 and -2) and then stimulated with recombinant LT\(\alpha\)\(\beta\). Both siRNAs for A20 were able to dramatically reduce the expression of A20 (Fig. 1a) and efficiently block the known function of A20; activation of the canonical pathway (phosphorylation of IKK and IkB\(\beta\)) by IL-1 in the late phase (120 min after stimulation) was significantly enhanced in these A20-knockdown cells (Supplementary Fig. S1^26). Ligation of LT\(\beta\)R resulted in the accumulation of NIK protein and the enhanced processing of p100 to p52 in the control siRNA-treated cells while these processes were significantly inhibited in A20-knockdown cells (Fig. 1a). NIK mRNA expression was not significantly affected by A20-1 siRNA (Fig. 1b). Although A20-2 siRNA slightly reduced (approx. 20%) NIK mRNA expression (Fig. 1b), this siRNA caused dramatic reduction (more than 80%) in NIK protein expression (Fig. 1a), indicating that the reduction in NIK mRNA accounts for little if any of the NIK protein reduction seen in the A20 knockdown cells. Therefore, we speculated that A20 regulates NIK protein expression at the translational or post-translational level. The specificity of this knockdown experiment was confirmed by a rescue experiment in which an siRNA A20-1-sensitive, A20-2-resistant A20 expression construct was introduced into MDA-MB-231 cells (Fig. 1a). Furthermore, LT\(\beta\)R-stimulation failed to induce NIK accumulation and efficient p100 processing in A20\(\sim\)-mouse embryo fibroblasts (MEFs), whereas their robust induction was observed in wild-type MEFs (Fig. 1c). Retrovirus-mediated introduction of A20 into A20\(\sim\) MEFs restored the LT\(\beta\)R-induced NIK accumulation and p100 processing (Fig. 1d) without affecting NIK mRNA levels (Fig. 1e). LT\(\beta\)R-stimulation-induced nuclear translocation of p52 and RelB, major NF-kB subunits that are activated in the non-canonical pathway, was abolished in A20\(\sim\) MEFs but was restored by reintroducing A20 (Fig. 1d). Taken together, A20 is crucial for the efficient activation of the non-canonical NF-kB pathway induced by LT\(\beta\)R signaling.

We next sought to determine whether A20 is commonly involved in the non-canonical NF-kB pathways of various members of the TNF receptor superfamily (TNFRSF). Fibroblast growth factor-inducible 14 (Fn14)-induced NIK accumulation and p100 processing in response to stimulation with tumor necrosis factor-like weak inducer of apoptosis (TWEAK) were significantly reduced in the A20-knockdown MDA-MB-231 cells and A20\(\sim\) MEFs but were restored by their complementation with A20 (Fig. 2a). Similarly, efficient NIK accumulation and p100 processing induced by the stimulation of receptor activator of NF-kB (RANK) with RANK ligand (RANKL) depended on A20 (Fig. 2b). By contrast, CD40 stimulation similarly induced NIK accumulation in the presence or the absence of A20 (Fig. 2c). Taken together, these results strongly suggest that A20 acts as a positive regulator for most but not all of the non-canonical NF-kB pathways. CD40 may have an A20-independent regulatory mechanism for its non-canonical pathway.

**Overexpression of A20 leads to activation of the non-canonical NF-kB pathway.** To elucidate the function of A20 in the non-canonical pathway, the expression levels of proteins involved in the non-canonical pathway were compared between control and TAP-A20-overexpressing HEK293T cells used for the initial analysis of the A20-containing complexes. Interestingly, A20 overexpression resulted in a significant enhancement of p100 processing (Fig. 3a) and an increase in NIK accumulation (Fig. 3b), although the mRNA level of NIK was not significantly affected (Fig. 3c). Given that NIK accumulation and p100 processing are induced in response to extracellular stimuli that activate the non-canonical pathway\(^{3,4}\), overexpression of A20 may trigger core molecular events required for the activation of the non-canonical pathway in the absence of extracellular stimuli. However, the degradation of TRAF3 observed in the activation of the non-canonical pathway induced by extracellular stimulation did not occur as a result of A20 overexpression (Fig. 3a), while TRAF3 degradation was similarly induced upon LT\(\beta\)R stimulation in the presence or absence of A20 (Fig. 3c). Given that stimulation-induced NIK accumulation was not observed in A20\(\sim\) MEFs, these results suggest that ubiquitination and subsequent degradation of TRAF3 could occur without attenuation of NIK ubiquitination.

cIAP1/2 are ubiquitin ligases of NIK, whereas TRAF2/TRAF3 heterodimers act as the molecular bridge that recruits cIAP1/2 to NIK, thereby forming the cIAP1/2-TRAF2-TRAF3-NIK complex to conjugate the K48 polyubiquitin chain to NIK. This polyubiquitination results in constitutive proteasomal degradation of NIK in unstimulated cells\(^{3,4,12}\). Upon stimulation, cIAP1/2 may no longer recognize NIK as a substrate, thereby inducing NIK accumulation. Given that A20 is likely to regulate NIK protein expression translationally or post-translationally, we first checked whether A20 regulates the stability of NIK protein. MDA-MB-231 cells transfected with either A20-2 siRNA or control siRNA were treated with MG132, a proteasome inhibitor, to induce NIK accumulation. The
Figure 1 | A20 is required for efficient activation of the non-canonical NF-κB pathway upon LTβR stimulation. (a) MDA-MB-231 cells infected with control virus or virus expressing siRNA A20-1-sensitive, A20-2-resistant human A20 were transfected with the indicated siRNAs. After 72 hr, the cells were untreated or treated with recombinant LTβR2 (500 ng/ml) for 3 hr. Cell lysates were then prepared and subjected to immunoblotting with the indicated antibodies. Amounts of NIK and ratios of p52 to p100 are shown as relative values, with the corresponding values from the unstimulated control siRNA-transfected cells set to 1. (b) MDA-MB-231 cells were transfected with the indicated siRNAs. After 72 hr, total RNA was extracted and the expression levels of NIK (MAP3K14) mRNA were measured by real-time RT-PCR. The level of GAPDH mRNA expression was used to normalize the data. The expression level of NIK mRNA in control cells was set to 1. (c) and (d) A20+/+ MEFs and A20−/− MEFs (c) or A20+/+ MEFs or A20−/− MEFs infected with control virus or virus expressing mouse wild type A20 (d) were stimulated with an agonistic anti-LTβR mAb (1 μg/ml) for the indicated time. Cell lysates were prepared and subjected to immunoblotting as in (a). (e) Total RNA was extracted from A20−/− MEFs infected with control virus or virus expressing mouse wild type A20, and real-time RT-PCR was performed as in (b), except that the β-actin mRNA expression level was used to normalize the data. The NIK mRNA expression level in A20−/− MEFs infected with control virus was set to 1. (f) A20−/− MEFs infected with control virus or virus expressing mouse wild type A20 were either unstimulated or stimulated with an agonistic anti-LTβR mAb (1 μg/ml) for 5 hr. Cells were then fractionated into cytoplasmic and nuclear fractions for subsequent immunoblotting as in (a). Tubulin is shown as a cytoplasmic marker, and PARP-1 is shown as a nuclear marker. The results shown in (b) and (e) indicate the mean ± SD (n = 3). The depicted results are representative of three independent experiments.
cells were then further incubated with MG132-free media supplemented with cycloheximide, a protein synthesis inhibitor, to analyze NIK protein stability. A20 knockdown shortened the half-life of the NIK protein (Supplementary Fig. S2), suggesting that A20 is likely to stabilize NIK by interacting with the NIK ubiquitin-ligase complex composed of cIAP1/2, TRAF2, and TRAF3. To elucidate how A20 is involved in the attenuation of the interaction between NIK and cIAP1/2, TAP-A20-containing protein complexes were purified and components of the complex were analyzed. cIAP1 and TRAF2 but not cIAP2 and TRAF3 were co-precipitated with TAP-A20 (Fig. 3d). cIAP2 was not detected in the complex, most likely because of its low expression in HEK293T cells. However, the lack of TRAF3 in the A20-containing complex may suggest that association of A20 with either cIAP1/2 or TRAF2 results in the dissociation of the TRAF2/TRAF3 heterodimer, as confirmed and discussed below. To determine if a similar A20-containing complex forms under physiological conditions, endogenous A20 was immunoprecipitated in MDA-MB-231 cells before or after 3 h of LTα1β2 stimulation.

Although A20 was co-precipitated with cIAP1/2 and TRAF2 in unstimulated cells, the amounts of cIAP1/2 and TRAF2 included in the A20-containing complex increased significantly with a concomitant increase in A20 upon stimulation induced by the canonical NF-κB pathway (Fig. 3e). By contrast, A20 was not associated with either TRAF3 or NIK (Fig. 3e), as demonstrated in the A20 overexpression experiment (Fig. 3d).

The ZnF7 of A20 is crucial for cIAP1 binding and NIK stabilization. Because A20 consists of an N-terminal OTU domain with deubiquitinase activity and a C-terminal ZnF region with E3 ligase activity, we next determined which domain binds to the cIAP1/2, TRAF2, and TRAF3. To elucidate how A20 is involved in the attenuation of the interaction between NIK and cIAP1/2, TAP-A20-containing protein complexes were purified and components of the complex were analyzed. cIAP1 and TRAF2 but not cIAP2 and TRAF3 were co-precipitated with TAP-A20 (Fig. 3d). cIAP2 was not detected in the complex, most likely because of its low expression in HEK293T cells. However, the lack of TRAF3 in the A20-containing complex may suggest that association of A20 with either cIAP1/2 or TRAF2 results in the dissociation of the TRAF2/TRAF3 heterodimer, as confirmed and discussed below. To determine if a similar A20-containing complex forms under physiological conditions, endogenous A20 was immunoprecipitated in MDA-MB-231 cells before or after 3 h of LTα1β2 stimulation.
A20−/− MEFs, neither mutant could compensate for the impaired activation of the non-canonical pathway in A20−/− MEFs (Fig. 4c), indicating that the ZnF domain can associate with the cIAP1/TRAF2 complex but that the OTU domain is additionally required for activation of the non-canonical pathway. To further explore the role of A20 in the non-canonical pathway, we next addressed whether the catalytic activities of A20 are required for activation of the non-canonical pathway. An OTU domain mutant (A20-OTUmt) that lacks deubiquitinate activity because the catalytic Cys-103 was substituted with Ala and the fourth ZnF mutant (A20-ZnF4mt), which lacks E3 ligase activity because its conserved Cys residues (Cys-609, Cys-612) are substituted with Ala (Fig. 4a), were stably expressed in A20−/− MEFs. LTβR stimulation-dependent NIK accumulation and p100 processing were similarly induced in cells expressing these mutants and A20-WT (Fig. 4d), indicating that neither the deubiquitinate nor the E3 ligase activity is required for the LTβR-induced activation of the non-canonical pathway. Because a recent study revealed that the seventh ZnF (ZnF7) domain of A20 is crucial for the binding of a K63 or linear polyubiquitin chain, thereby suppressing the canonical NF-κB pathway, we analyzed A20−/− MEFs expressing the ZnF7 mutant (A20-ZnF7mt) in which the conserved Cys residues (Cys-764, Cys-767) of ZnF7 are substituted with Ala (Fig. 4a). Expression of A20-ZnF7mt did not restore the non-canonical pathway in A20−/− MEFs (Fig. 4d). In addition, the A20-ZnF7mt and the A20-E661X, in which the nonsense mutation reported in diffuse large B-cell lymphoma results in the generation of...
Figure 4 | ZnF7 of A20 is crucial for the association of A20 with the cIAP1/TRAF2 complex and efficient activation of the non-canonical NF-κB pathway. (a) A schematic representation of various human and mouse A20 mutants used in this study. (b) and (e) The cell lysates prepared from HEK293T cells infected with control virus or virus expressing TAP-A20 or its mutant were subjected to affinity pull-down with Strep-Tactin Superflow beads. The precipitates were subjected to immunoblotting. (c) (a) and (d) A20−/− MEFs infected with control virus or virus expressing wild type A20 or its mutant were stimulated with an agonistic anti-LTβR mAb (1 μg/ml) for the indicated times. Cell lysates were prepared and subjected to immunoblotting with the indicated antibodies. Quantitative ratios of p52 to p100 were calculated based on the data and are shown as ratios relative to those of the unstimulated control virus-infected cells. The depicted results are representative of three independent experiments.
Direct binding of A20 and cIAP1/2 results in the dissociation of the TRAF2/TRAF3 heterodimer. Although several models for the stimulation-dependent stabilization of NIK have been proposed\(^{23,24,25}\), the critical event common to these models is the attenuation of the cIAP1/2-catalyzed K48 polyubiquitination of NIK resulting from the dissociation of the c-IAP1/2/TRAF2/TRAF3/NIK complex. Therefore, we next addressed whether A20 expression has any effect on complex formation because A20 overexpression triggers activation of the non-canonical pathway and concurrently induces the association of A20 with the cIAP1/TRAF2 complex (Fig. 3a and d). N-terminally TAP-tagged NIK, alone or together with either Mmc-A20-WT or Mmc-A20-ZnF7mt, was stably expressed in HEK293T cells, and the NIK-associated proteins were analyzed by pull-down of TAP-NIK. TAP-NIK was associated with cIAP1, TRAF2, and TRAF3 in parental cells. The association of NIK with cIAP1 and TRAF2 but not with TRAF3 was significantly reduced by the over-expression of A20-WT but not A20-ZnF7mt (Fig. 5a). These results strongly suggest that the binding of A20 to cIAP1 through ZnF7 of A20 resulted in the dissociation of the complex by reducing the interaction between TRAF2 and TRAF3, thereby suppressing polyubiquitination and inducing the accumulation of NIK.

To further understand the mechanism of A20-mediated activation of the non-canonical pathway, the interaction between A20 and the cIAP1/TRAF2 complex was analyzed more precisely. We first attempted to determine which component of the cIAP1/TRAF2 complex binds A20. MV-1, a second mitochondria-derived activator of caspase (SMAC) mimetic that induces the depletion of cIAP1 by activating its auto-ubiquitination\(^{26}\), significantly inhibited the interaction of A20 with TRAF2 (Fig. 5b). Given that cIAP1 directly binds TRAF2 (Ref. 27), this result strongly suggests that A20 indirectly associates with TRAF2 via cIAP1. Because ZnF7 of A20 has been shown to play a critical role in the binding of A20 to K63 or linear polyubiquitin chains, we next addressed whether the binding between A20 and cIAP is direct or requires ubiquitin. Glutathione-S-transferase (GST) was fused to the fragment of A20 (aa 647–790) encompassing the ZnF5 to the ZnF7 (GST-A20-ZnF5–7), to the same fragment carrying the C779A/C782A mutation to inactivate the ZnF7 (GST-A20-ZnF5–7mt) (Fig. 5c), and to the whole cIAP1 (GST-cIAP) and expressed in E. coli (Supplementary Fig. S3). Because E. coli does not produce ubiquitin, recombinant proteins prepared from E. coli are not ubiquitinated or co-purified with ubiquitin. Bead-bound GST-cIAP1 was then cleaved with 3C protease to release cIAP1 (Supplementary Fig. S3), which was then mixed with GST-A20-ZnF5–7 or its mutant and followed by a GST pull-down assay. cIAP1 directly bound GST-A20-ZnF5–7 but not GST-A20-ZnF5–7mt. cIAP was also pulled down when GST-A20-ZnF5–7 was mixed with GST-A20-ZnF5–7mt, indicating that the degradation products of GST-A20-ZnF5–7mt did not inhibit the interaction between cIAP1 and A20-ZnF5–7 (Fig. 5d). These results indicate that A20 directly binds to cIAP1/2 in the absence of ubiquitin. To determine the domain of cIAP1 to which A20-ZnF5–7 binds, TAP-tagged cIAP1 and its deletion mutants were expressed in HEK293T cells and partially purified with Strep–Tactin beads (Fig. 5c). These beads were then incubated with GST-A20-ZnF5–7 proteins, and pull-down assays were performed to assess the association between the cIAP1 deletion mutants and GST-A20-ZnF5–7. The baculovirus IAP repeat 2 (BIR2) and BIR3 domains are each required for the binding of cIAP1 to A20, and together, they are sufficient for binding (Fig. 5e). Given that TRAF2 binds the BIR1 domain\(^{28}\), these results are consistent with the formation of the A20/cIAP1/TRAF2 complex before and after LTβR stimulation (Fig. 3e).

K63 polyubiquitin, but not linear polyubiquitin, and cIAP1 competitively bind to A20. ZnF7 has been reported to bind to either K63 polyubiquitin or linear polyubiquitin\(^{20–22}\). Given that these polyubiquitin chains are generated during the activation of the canonical pathway\(^2\), one may speculate that these polyubiquitin chains modulate the activation of the non-canonical pathway by competing against cIAP1/2 upon binding to A20. We first assessed which of the two chain types could bind efficiently to ZnF7 by performing the GST pull-down assay after incubation of GST-A20-ZnF5–7 with recombinant polyubiquitin chains. In contrast to the previous report\(^{23,24}\), K63 polyubiquitin binds to A20 more efficiently than the linear polyubiquitin chain (Fig. 6a). Moreover, the addition of recombinant K63 polyubiquitin chains but not linear polyubiquitin chains inhibited the interaction between ZnF7 and cIAP1 (Fig. 6b), indicating that cIAP1 and K63 polyubiquitin chains bind to ZnF7 competitively. These results suggest a novel regulatory role of the K63 polyubiquitin chain in the non-canonical NF-κB pathway.

Discussion

Although only a specific subset of TNFRSF activates the non-canonical NF-κB pathway, this pathway plays critical and unique roles in establishing the immune system. In general, the canonical pathway rapidly activates the p50/RelA heterodimer, whereas the non-canonical pathway slowly activates the p52/RelB heterodimer afterwards (Fig. 7a and b). Due to these differences, the two pathways have distinct target gene specificities and gene expression kinetics, which are crucial for the proper regulation of inflammation and immune responses\(^{29}\). Therefore, elucidation of the molecular mechanism by which the non-canonical pathway becomes activated is of particular interest. A critical event that triggers the non-canonical pathway is the attenuation of NIK degradation, resulting from K48 polyubiquitination of NIK catalyzed by the NIK ubiquitin ligase complex, which includes TRAF2, TRAF3, and cIAP1/2. Genetic deficiency in any component of the ligase complex leads to the stabilization of NIK and activation of the non-canonical pathway\(^{29–31}\). In this paper, we clearly demonstrate that A20-deficiency significantly abrogates NIK accumulation and p100 processing to p52 that were induced by stimulation of LTβR, Fn14, and RANK. Furthermore, shifting from the canonical to the non-canonical pathway in LTβR signaling is not properly executed in A20 knockdown cells (Fig. 7a and b). These results strongly suggest that A20 is involved in the molecular switch that promotes the transition from canonical to non-canonical activation (Fig. 7c). Therefore, A20 is a double-edged sword: it is a positive regulator of the non-canonical pathway due to its involvement in the attenuation of NIK degradation as well as a negative regulator of the canonical pathway as identified previously.

The deubiquitinase and ubiquitin ligase activities of A20 are not required for the A20-mediated enhancement of non-canonical NF-κB activation, but ZnF7 is essential. In this sense, our observation is similar to the previously reported noncatalytic mechanisms of IKK inhibition by A20 in the canonical pathway\(^{30–32}\). However, one of the critical findings in this study is that ZnF7 binds cIAP1/2 in addition to its previously recognized binding to either K63 or linear polyubiquitin chains. Interestingly, the binding of cIAP1/2 to A20 was competitively blocked by the K63 polyubiquitin chain, suggesting that unanchored or substrate-conjugated K63 polyubiquitin chains generated during the activation of the canonical pathway may block the interaction of A20 with cIAP1/2, thereby blocking early activation of the non-canonical pathway.

How is A20 involved in the inactivation of the NIK ubiquitin ligase complex? Our results suggest that interaction of A20 with cIAP1/2 may lead to the dissociation of the TRAF2/TRAF3 interaction within...
Based on previous literature, the BIR1 domain of cIAP1/2 binds to the coiled-coil domain of TRAF2 (Ref. 27), which partially overlaps the TRAF3 binding domain in TRAF2 (Ref. 33). Moreover, our data indicate that ZnF7 of A20 binds to the BIR2 and BIR3 domains, which are adjacent to the BIR1 domain. Therefore, the interaction of A20 with cIAP1/2 may inhibit the TRAF2/TRAF3 interaction through structural changes in TRAF2 induced by the indirect interaction of A20 with TRAF2 via the BIR domains of cIAP1/2 or by the direct interaction between A20 and TRAF2, as suggested by the yeast two-hybrid experiment34.

Overexpression of A20 induced by transient transfection of the TAP-A20 expression vector into HEK293T cells was able to induce p100 processing and NIK accumulation without stimulation. Moreover, several hours after LTßR stimulation, A20 expression was significantly augmented with concomitant accumulation of NIK. These results might suggest that the accumulation of A20 upon

Figure 5 | Direct binding of A20 to cIAP1 results in the dissociation of the TRAF2/TRAF3 interaction. (a) HEK293T cells were infected with either control virus or virus expressing TAP-NIK. The TAP-NIK expressing cells were further infected with control virus or virus expressing human wild type A20 or its mutant. Cell lysates were prepared and subjected to affinity pull-down with Strep-Tactin Superflow beads. The lysates and precipitates were subjected to immunoblotting. (b) HEK293T cells were infected with either control virus or virus expressing TAP-A20. The cells were untreated or treated with MV-1, a SMAC mimetic (5 μM), for 5 hr. The cell lysates were prepared and subjected to immunoblotting. (c) A schematic representation of various mutants of GST-human A20 and TAP-human cIAP1. (d) Various combinations of recombinant GST-A20-ZnF5-7, GST-A20-ZnF5-7mt, and cIAP proteins were incubated for 3 hr, and the reaction mixtures were subjected to pull-down assay. The precipitates were analyzed by immunoblotting with the indicated antibodies. (e) TAP-tagged cIAP1 or its mutant was transiently expressed in HEK293T cells and precipitated with Strep-Tactin Superflow beads. The precipitated beads were suspended and incubated with GST-A20-ZnF5-7 protein for 3 hr. The beads were then washed and subjected to immunoblotting. The depicted results are representative of three independent experiments. See also Supplementary Fig. S3.

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stimulation by LTβR could be sufficient to trigger the non-canonical pathway, thereby resulting in slower kinetics of the non-canonical pathway in comparison with that of the canonical pathway. However, the following observations indicate that this is not the case: first, TNFα or IL-1 stimulation does not activate the non-canonical pathway, while both can induce A20 efficiently; second, the non-canonical pathway was activated with slow kinetics similar to that observed in wild-type MEFs and even in A20−/− MEFs complemented with A20 in which a sufficient amount of A20 is constitutively expressed irrespective of LTβR stimulation. These results indicate that the A20 accumulation in response to physiological stimulation is not sufficient and that other cooperative signal(s) triggered by the specific subset of TNFRSF is/are required for non-canonical activation.

It has been reported that stimulation-induced TRAF3 degradation is involved in NIK accumulation. In addition, both the activation
Figure 7 | A20 promotes switching from the canonical to the non-canonical NF-κB pathway upon LTβR stimulation. (a) MDA-MB-231 cells were transfected with either control siRNA or A20 siRNA (A20-2). After 72 hr, the cells were stimulated with recombinant LTαβ (500 ng/ml) for the indicated time. Whole cell lysates and nuclear extracts were then prepared and subjected to immunoblotting with the indicated antibodies.

(b) Quantitative ratios of phospho-IκBα to total IκBα (upper left), p52 to p100 (upper right), RelA to PARP-1 (lower left), and RelB to PARP-1 (lower right) were calculated based on the data shown in (a) and are shown as ratios relative to those of the unstimulated control siRNA-transfected cells. The depicted results are representative of three independent experiments. (c) A schematic model illustrating the A20-mediated switching from the canonical to the non-canonical NF-κB pathway (see the text for the details).
of the non-canonical LTβR pathway and TRAF3 degradation require receptor internalization\(^{25}\). However, we demonstrated that overexpression of A20 does not induce TRAF3 degradation but does induce non-canonical activation. Moreover, stimulation-induced TRAF3 degradation was not affected in the absence of A20. Therefore, TRAF3 degradation and receptor internalization are independent of A20 and may not be required for non-canonical activation at least in the cells used in this paper. Co-operation of A20-mediated destabilization of the TRAF2/TRAF3 complex and the A20-independent but receptor stimulation-dependent event(s) that are somehow associated with TRAF3 degradation could be essential for non-canonical activation in response to physiological stimulation. It has been reported that LTβR and NIK bind to TRAF3 at the same site and that LTβR stimulation results in the recruitment of TRAF3 to the oligomerized LTβR, causing the competitive displacement of NIK from TRAF3 and preventing NIK degradation by cIAP1/2 (Ref. 25).

Gene-knockout experiments revealed an essential role for the non-canonical LTβR pathway in lymph node development\(^{26,27}\). However, although A20-deficient mice were shown to have multiorgan inflammation, severe cachexia, and premature lethality, defects in lymph nodes have not been reported\(^{28}\). This result may be a consequence of mild lymph-node defects resulting from the residual activation of the non-canonical LTβR pathway in the absence of A20. Therefore, precise analysis of the conditional A20\(^{-/-}\) mice is required. Based on the analysis of the A20 gene in various tumors, it has been proposed that elevated activation of NF-κB by inactivation of A20 could be involved in cancer development\(^{28,29}\). By contrast, our results strongly suggest that excess expression of A20 could result in enhanced activation of the non-canonical NF-κB pathway, the excess activation of which is tightly linked to tumor formation\(^{1}\). We quantified the level of NF-κB activation in various breast cancer cell lines and reported that they were significantly high in triple-negative breast cancer, the most malignant type of breast cancer\(^{30}\). Based on our experimental data from DNA microarrays measuring non-canonical activation, seven of eight cell lines with the highest NF-κB activation show both constitutive activation of the non-canonical pathway and high expression of A20 (Supplementary Fig. S4). Therefore, activation or enhanced expression of A20, as well as its inactivation, is likely to cause tumor development in vivo. Although further experiments are required to support this hypothesis, our elucidation of the critical role of A20 may contribute to the identification and regulation of the NF-κB pathway and may facilitate the development of therapeutic strategies for various diseases caused by the dysregulation of NF-κB.

Methods

Reagents and plasmids. Recombinant human LTβ1/2 and TWEAK were purchased from R&D Systems. RANKL was purchased from Wako. Anti-mouse LTβR antibody was purchased from Enzo Life Sciences. MV1 was kindly provided by Y. Demizu (National Institute of Health Sciences, Japan). Human cDNAs encoding A20 (wild type and mutants), cIAP1 (wild type and mutants), CD40, and NIK and mouse cDNAs encoding A20 (wild type and mutants) and RANKL were generated by PCR and inserted into the retroviral vector pMXs, which was obtained from T. Kitamura (National Institute of Health Sciences, Japan). Human cDNAs encoding A20 (wild type and mutants), cIAP1, and anti-RelA, and anti-RelB (Santa Cruz Biotechnology); anti-NIK, anti-clIAP2, anti-TRAF2, anti-p100, anti-A20, anti-phospho-IκBα, anti-PARP1, and anti-phospho-IKK (Cell Signaling Technology); anti-tubulin and anti-p100 (Millipore); anti-cIAP1 (Enzo LifeSciences); anti-A20 (Bioscience); and anti-FLAG (Sigma-Aldrich); and horseradish peroxidase–conjugated secondary antibodies (GE Healthcare). For quantification, bands were analyzed with Image Lab 3.0 software (Bio-Rad).

GST pull-down assay. Human cDNAs encoding A20 ZnF5-7, ZnF5-7mt, and cIAP1 were generated by PCR and inserted into pGEX-4T-1. GST- and GST-fused proteins were expressed in E. coli DH5α and purified using Glutathione Sepharose 4B (GE Healthcare). The GST tag was removed from clIAP1 by Turbo3C protease (Wako). GST-ZnF5-7 proteins were dialyzed with PBS, K48-linked and K63-linked polyubiquitin chains (UbK48-7 and UbK63-7) were purchased from Biozol Biochem. Linear polyubiquitin chains (UbK48-7) were purchased from Enzo LifeSciences. A quantity of 0.5 μg of polyubiquitin chains or 0.5 μg of cIAP1 proteins was incubated with 0.5 μg of GST or GST-ZnF5-7 and 10 μl of Glutathione Sepharose 4B in TNE buffer [20 mM Tris–HCl (pH 7.5), 130 mM NaCl, 1% Triton X-100, 1 mM EDTA, 2 mM Na3VO4, 10 mM NaF] containing 0.5 mM DTT and 0.1 mg/ml BSA for 3 hr. After incubation, the beads were washed three times with TNE buffer and then suspended in sample buffer. For competition experiments, 0.5 μg of cIAP1 protein was incubated with 0.5 μg of GST-ZnF5-7 and 10 μl of Glutathione Sepharose 4B in TNE buffer with 0.5 mM DTT and 0.1 mg/ml BSA for 3 hr, and then 0.5 or 3 μg of K63-linked polyubiquitin chains were added to the mixture. After a 3-hr incubation, the beads were washed three times with TNE buffer and then suspended in sample buffer.

Immunoprecipitation and Strept-Tactin pull-down. Cells were lysed in TNE buffer and centrifuged to remove cellular debris. The resulting supernatant was mixed with 1 μg of the appropriate antibody together with 10 μl of protein G Sepharose beads (GE Healthcare) or 10 μl of Strept-Tactin Superflow beads (IBA, USA). After overnight incubation, the beads were washed three times with TNE buffer and then suspended in sample buffer. For competition experiments, 0.5 μg of cIAP1 protein was incubated with 0.5 μg of GST-ZnF5-7 and 10 μl of Glutathione Sepharose 4B in TNE buffer with 0.5 mM DTT and 0.1 mg/ml BSA for 3 hr, and then 0.5 or 3 μg of K63-linked polyubiquitin chains were added to the mixture. After a 3-hr incubation, the beads were washed three times with TNE buffer and then suspended in sample buffer.

Tandem affinity purification and mass spectrometry. HEK293T cells expressing TAP-A20 were lysed in TNE buffer with protease inhibitor cocktail (Roche), and cell debris was removed by centrifugation at 10,000 × g for 15 min to prepare the cell lysate. Protein complexes containing A20 were precipitated with Strept-Tactin Superflow beads and eluted with desthiobiotin. The eluates were treated with anti-Flag M2 beads, and the protein complexes were eluted with Flag peptides. The eluted proteins were digested with trypsin and loaded onto an automated nanoflow liquid chromatography system (Dima) coupled to a linear ion trap orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific). The tandem mass spectrometry signals were processed against human protein sequences in the NCBI RefSeq database using the Mascot algorithm (Matrix Science).

Statistical analysis. Statistically significant differences between the mean values were determined using Student’s t-test. Data are presented as the means ± SD.

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