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XEDAR activates the non-canonical NF-κB pathway

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ABSTRACT

Members of the tumor necrosis factor receptor (TNFR) superfamily are involved in a number of physiological and pathological responses by activating a wide variety of intracellular signaling pathways. The X-linked ectodermal dysplasia receptor (XEDAR; also known as EDA2R or TNFRSF27) is a member of the TNFR superfamily that is highly expressed in ectodermal derivatives during embryonic development and binds to ectodysplasin-A2 (EDA-A2), a member of the TNF family that is encoded by the anhidrotic ectodermal dysplasia (EDA) gene. Although XEDAR was first described in the year 2000, its function and molecular mechanism of action is still largely unclear. XEDAR has been reported to activate canonical nuclear factor κB (NF-κB) signaling and mitogen-activated protein (MAP) kinases. Here we report that XEDAR is also able to trigger the non-canonical NF-κB pathway, characterized by the processing of p100 (NF-κB2) into p52, followed by nuclear translocation of p52 and RelB. We provide evidence that XEDAR-induced p100 processing relies on the binding of XEDAR to TRAF3 and TRAF6, and requires the kinase activity of NIK and IKKα. We also show that XEDAR stimulation results in NIK accumulation and that p100 processing is negatively regulated by TRAF3, cIAP1 and A20.

1. Introduction

The dimeric transcription factor nuclear factor-κB (NF-κB) forms a key component in several biological processes, ranging from immune and inflammatory signaling to cell survival and development [1]. The NF-κB family consists of five members: p50, p52, RelA (p65), c-Rel, and RelB, of which p50 and p52 are synthesized as larger precursors, called p105 (NF-κB1) and p100 (NF-κB2), respectively [2]. Receptor-mediated NF-κB activation comes in two flavors: the canonical (or classical) and the non-canonical (or alternative) pathway. The canonical NF-κB pathway is mainly involved in immunity and inflammation, and is triggered by several receptors, such as TNF receptor 1 (TNFR1), interleukin-1 receptor, Toll-like receptors (TLRs) and T cell receptor. Canonical NF-κB activation involves the activation of the Inhibitor of NF-κB kinase (IKK) complex, which consists of two catalytic subunits, IKKα and IKKβ, and the regulatory subunit IKKγ (also known as NEMO) [3]. IKKβ phosphorylates the NF-κB inhibitor IκBα leading to its Lys48-ubiquitination and proteasomal degradation. As a result, the NF-κB dimer (mostly p50/RelA) is set free and translocates to the nucleus, where it binds the promoter of NF-κB responsive genes.

The non-canonical NF-κB pathway differs from the canonical pathway in functional outcome, architecture and regulation. Non-canonical NF-κB signaling is elicited by TNFR subfamily members such as CD40, lymphotoxin-β receptor (LTβR) and B-cell activating factor receptor (BAFF-R), with specific functions in lymphoid organ development and B cell survival [2,4]. At the molecular level, the non-canonical NF-κB pathway is characterized by the activation of inactive p100/RelB complexes in the cytoplasm via proteolytic processing of p100 to p52, resulting in the nuclear translocation of

Abbreviations: BAFF-R, B-cell activating factor receptor; CD40, cluster of differentiation 40; cIAP, cellular inhibitor of apoptosis; DM, double mutant; DUBA, deubiquitinating enzyme A; EDA, ectodysplasin; EDAR, EDA receptor; Edaradd, EDAR associated death domain; HRP, horse radish peroxidase; IκB, inhibitor of κB; IKK, inhibitor of NF-κB kinase; KI, kinase inactive; LTβR, lymphotoxin β receptor; Lys, lysine; MAP, mitogen-activated protein; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; NIK, NF-κB inducing kinase; RANK, receptor activator of NF-κB; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Ser, serine; SR, superrepressor; TL, total lysate; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNFR associated factor; XEDAR, X-linked ectodermal dysplasia receptor; WT, wild type.

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p52/RelB [5–7]. This pathway evolves independent of IKKβ and NEMO, but requires the activity of IKKα and NF-κB inducing kinase (NIK) [8,9]. In resting cells NIK levels are kept low by TNFR associated factor 3 (TRAF3), which promotes constitutive proteasomal degradation of NIK by virtue of the E3 ubiquitin ligase complex consisting of TRAF2-TRAF3-cellular inhibitor of apoptosis (cIAP)1 and 2 [10–12]. Upon receptor stimulation, NIK is stabilized due to the cIAP-mediated degradation of TRAF3 [4]. NIK then activates and also promotes binding of IKKα to its substrate p100, which is ubiquitinated and processed to p52 upon phosphorylation [8,9].

The X-linked ectodermal dysplasia receptor (XEDAR; also known as EDA2R or TNFRSF27) is a member of the TNFR superfamily that is recognized by ectodysplasin-A2 (EDA-A2), which is generated by alternative splicing of the EDA receptor (EDAR) ligand EDA-A1. EDA-A1 differs from EDA-A2 by the presence of two extra amino acids [13,14]. Mutations in EDA, EDAR, Edaradd (adapter protein required for EDAR signaling), and NEMO can cause hypohidrotic (or anhidrotic) ectodermal dysplasia and X-linked anhidrotic ectodermal dysplasia with immunodeficiency, characterized by absence or sparsity of hair (alopecia), missing or misshapen teeth, and absent or reduced sweat glands [15]. Similarly, TRAF6 deficient mice display hypohidrotic ectodermal dysplasia [16]. TRAF6 does not bind EDAR, but does associate with XEDAR and mediates XEDAR-induced activation of the canonical NF-κB pathway [16]. Despite the large similarity between EDAR and XEDAR, limited cases of a mutation in the XEDAR gene have been reported in patients with androgenic alopecia or with mild symptoms of hypohidrotic ectodermal dysplasia [15].

Although XEDAR was already described 15 years ago [14], its function and signaling mechanisms are still largely unclear. XEDAR can activate the canonical NF-κB pathway, which involves its direct interaction with TRAF3 and TRAF6 [14,17]. Here we show that XEDAR can also activate the non-canonical NF-κB signaling pathway. The latter requires binding of TRAF3 and TRAF6 to the intracellular domain of XEDAR. XEDAR-induced non-canonical NF-κB signaling involves NIK and IKKα, and is negatively regulated by TRAF3, cIAP and A20.

2. Materials and methods

2.1. Cells and reagents

HEK293T 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. Sepharose beads coated with protein G were from Sanbio B.V. (Uden, Netherlands), trisacryl beads coated with protein A were from Pierce (Rockford, USA), and recombinant human EDA-A2 was from R&D Systems (Abingdon, UK). Monoclonal anti-Myc was produced in house. Commercial antibodies: anti-E-tag (Abcam, Cambridge, UK); anti-FLAG M2 (Sigma, St-Louis, MO, USA); anti-p52 (Bio-Connect, Huissen, Netherlands); anti-actin (MP Biomedicals, Illkirch, France); anti-NIK (clone H248) (Santa Cruz, California, USA); anti-IKKα (clone B8) (Santa Cruz, California, USA); anti-HA (Eurogentec, Liège, Belgium); anti-TRAF3 (clone H20 and G6) (Santa Cruz, California, USA); anti-lamin (Cell Signaling, Massachusetts, USA); anti-tubulin (Sigma, St-Louis, MO, USA); anti-RelB (clone C19) (Santa Cruz, California, USA); anti-mouse and anti-rabbit immunoglobulin antibody conjugated with Dylight 680 or 800 (Pierce, Rockford, USA) or with HRP (Amersham, Diegem, Belgium).

2.2. Expression plasmids

pCAGGS-E-hA20 (LMBP3778), pCAGGS-FLAG-DUBA (LMBP 6414), pCAGGS-FLAG-IKKα KI (LMBP5484), and pEF6-E-TRAF2 (LMBP5245) 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/about-us/bccm-lmbp). The following plasmids were gifts: pcDNA3-HA-IκBα SR (Dr. J. Piette, University of Liège, Belgium); pcDNA3-NIK KI mutant (Dr. D. Wallach, Weizmann Institute of Science, Rehovot, Israel); pcDNA3.1-FLAG-TRAF3 (Dr. S. Pype, KUL, Leuven, Belgium); pMT2T-p100 WT and mutants (Dr. A. Chariot, University of Liège, Belgium). pcDNA3.1-Myc-cIAP1 WT was obtained from Addgene (Cambridge, USA). pCAGGS-E-mXEDAR WT was made by recloning the mouse XEDAR coding sequence in pCAGGS-E. The mutants were generated by PCR mutagenesis.

2.3. Nuclear and cytoplasmic cell fractions

HEK293T cells were seeded at 2×10^5 in 6-well plates and transfected with 1 μg total DNA of various constructs. The day after, cells were washed twice in PBS, harvested and incubated in 300 μl B1 buffer (10 mM HEPES pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA and 0.5 mM DTT) for 15 min on ice. 15 μl of a 10% NP40 solution was added, followed by mild vortexing. After full speed centrifugation (14000 rpm) for 15 min in an eppendorf microcentrifuge, cytoplasmic fractions were collected. Pellets were washed in B1 buffer and incubated for 15 min with 50 μl B2 buffer (10 mM HEPES pH 7.5, 1% NP40, 10 mM KCl, 1 mM MgCl2, 400 mM NaCl, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA and 0.5 mM DTT). Nuclear fractions were collected after full speed centrifugation for 15 min. Both B1 and B2 buffers were supplemented with protease inhibitors (2.1 μM leupeptin, 0.15 μM aprotinin, 1 mM pefabloc) and phosphatase inhibitors (200 μM sodium-orthovanadate, 10 mM sodium fluoride, 5 μg/ml β-glycerophosphate) throughout the whole procedure. Protein concentration of both fractions was measured by Bio-Rad Protein Assay (according to manufacturer's protocol) before fractionation by SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane, and probed with 1:1000 dilution of the indicated antibodies.

2.4. Immunoprecipitation and western blot analysis

HEK293T cells were seeded at 1.2×10^6 on 90-mm plates and transfected with 5 μg total DNA of various constructs. The day after, cells were harvested and lysed in E1A buffer (50 mM HEPES pH 7.6, 250 mM NaCl, 5 mM EDTA and 0.5% NP-40) in the case of TRAF3 detection or RIPA buffer (50 mM Tris–HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40 and 0.25% sodiumdeoxycholate) in the case of NIK detection, supplemented with protease and phosphatase inhibitors as described above throughout the whole procedure. After full speed centrifugation for 15 min, nine tenths of the lysate was incubated with respectively 1 μg anti-TRAF3 or anti-NIK antibody for 1 h at 4 °C, mixed with 15 μl of protein G-sepharose (in case of TRAF3) or protein A-trisacryl beads (in case of NIK), and incubated for an additional 2 h. The beads were washed four times with E1A (in case of TRAF3) or RIPA buffer (in case of NIK). The immunoprecipitates as well as the remainders of each cell lysate were fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with 1:1000 dilution of the indicated antibodies.

3. Results and discussion

3.1. XEDAR activates non-canonical NF-κB signaling

To investigate if XEDAR is able to activate the non-canonical NF-κB pathway, we analyzed its ability to induce p100 processing to

p52 along with p52/RelB nuclear translocation in HEK293T cells that were transfected with a XEDAR expression plasmid and stimulated for increasing times with recombinant EDA-A2. Treatment with EDA-A2 significantly induced p100 processing and nuclear translocation of p52/RelB, which was most pronounced after 8 h stimulation (Fig. 1A). Overexpression of XEDAR as such has been shown to activate the canonical NF-κB pathway [17]. Similarly, overexpression of XEDAR in the absence of EDA-A2 also activated non-canonical NF-κB signaling, which was further increased by EDA-A2 stimulation. In subsequent experiments we therefore sticked to overexpression of XEDAR to induce non-canonical NF-κB signaling. To rule out that increased p52 levels are merely a consequence of increased p100 expression due to the ability of XEDAR to induce canonical NF-κB signaling [18], we studied XEDAR-induced p100 processing in cells overexpressing IκBα(S32A/S36A) (also known as IκBα superrepressor (SR)), which prevents canonical NF-κB signaling. This showed that XEDAR-induced p100 processing is independent of the canonical NF-κB pathway (Fig. 1B). To conclude, these data demonstrate that XEDAR is able to induce non-canonical NF-κB signaling.

3.2. Mutation of TRAF3/6 binding sites in XEDAR abolishes its ability to activate non-canonical NF-κB signaling

The intracellular domain of XEDAR has been shown to interact with TRAF1, TRAF3 and TRAF6. Studies with TRAF-binding deficient XEDAR mutants demonstrated a requirement for TRAF3 and TRAF6 binding in canonical NF-κB activation [17]. We similarly generated XEDAR mutants that are deficient in binding of TRAF3 (T3*; E232K), TRAF6 (T6*; E256K) or both (DM) and tested their ability to activate non-canonical NF-κB activation upon overexpression. We made use of cells expressing the IκBα SR, excluding a possible role of differences in canonical NF-κB activation and increased p100 levels. Single mutation in the TRAF3 binding site did not significantly diminish XEDAR-induced p100 processing, while mutation of the TRAF6 binding site abolished the response partially. Mutation of both binding sites (DM) almost completely prevented XEDAR-induced p100 processing (Fig. 2). Together, these data support the notion that binding of TRAF3 and TRAF6 to the intracellular domain of XEDAR is required for proper XEDAR-induced non-canonical NF-κB activation.

3.3. XEDAR-induced p100 processing requires NIK and IKKα kinase activity

Non-canonical NF-κB signaling by other members of the TNFR superfamily requires NIK and IKKα kinase activity [8,9]. We therefore investigated the potential role of NIK and IKKα in XEDAR-induced p100 processing by analyzing the effect of overexpression of kinase-inactive (KI) NIK and IKKα mutants, which

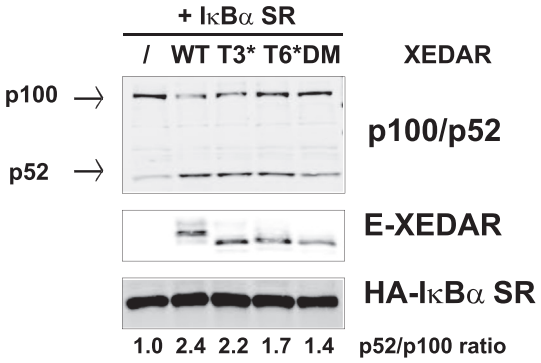

Fig. 2. Efficient non-canonical NF-κB activation triggered by XEDAR requires interaction of XEDAR with TRAF3 and TRAF6. HEK293T cells were transiently transfected with E-tagged versions of wild-type XEDAR (WT) or XEDAR mutants defective in binding for TRAF3 (T3*), TRAF6 (T6*), or both (DM), as well as HA-tagged IκBα SR. p52 formation was determined by immunoblotting with anti-p100/p52 antibody, expression of XEDAR proteins and IκBα SR was detected by anti-E-tag or anti-HA-tag antibodies, respectively.

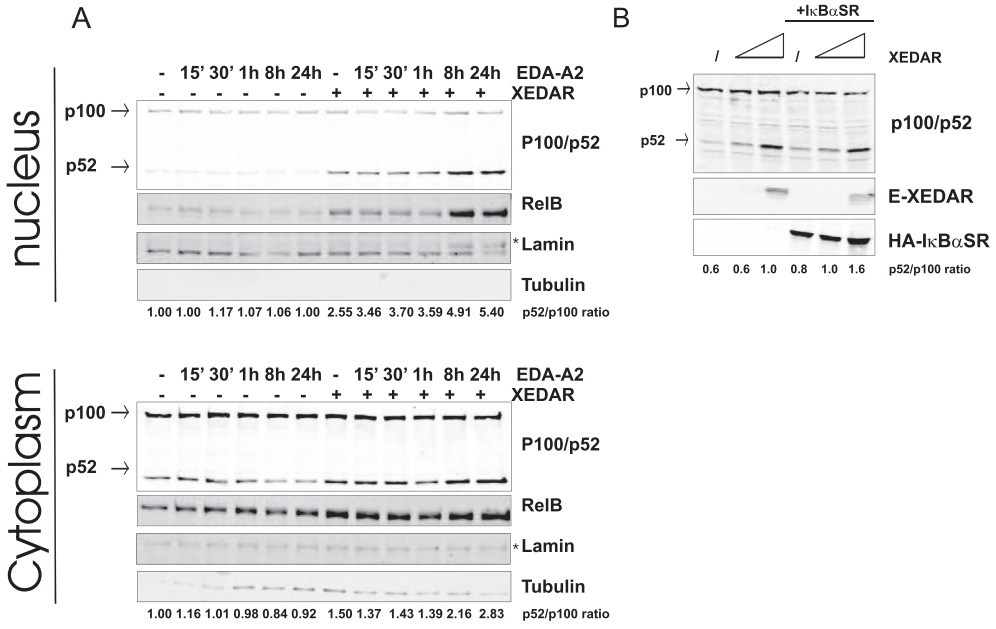

Fig. 1. Induction of the non-canonical NF-κB pathway by XEDAR – EDA-A2. A. Induction of p100 processing and nuclear accumulation of p52/RelB upon XEDAR expression and EDA-A2 stimulation. HEK293T cells were transiently transfected with empty vector or XEDAR. 24 h later, cells were treated with EDA-A2 (10 ng/ml) for the indicated times. Nuclear (top) and cytoplasmic cell fractions (bottom) were made and analyzed by immunoblotting with p100/p52 and RelB antibodies (blots were reprobed with lamin and tubulin antibodies as a control for nuclear and cytoplasmic fractions, respectively; * indicates the signal for RelB that was left after reprobing the anti-RelB blot with anti-lamin). B. XEDAR-induced p100 processing is independent of the canonical NF-κB pathway. HEK293T cells were transiently transfected with E-tagged XEDAR in combination with a plasmid encoding HA-tagged IκBα SR or empty vector as control. After 24 h, cells were lysed and p100 processing was determined by immunoblotting with anti-p100/p52.

behave as dominant-negative mutants, on XEDAR-induced p100 processing in HEK293T cells. Both kinase mutants strongly inhibited XEDAR-induced formation of p52 (Fig. 3A), indicating a role for NIK and IKKα in XEDAR-induced non-canonical NF-κB signaling.

p100 processing requires phosphorylation in its PEST domain by NIK (at Ser866 and Ser870) and IKKα (at Ser872) [4,18]. We therefore compared the ability of XEDAR overexpression to induce processing of wild-type p100 and p100 mutants in which the above mentioned phosphorylation sites were mutated (S866A, S870A and S872A). In contrast to wild-type p100, none of the p100 mutants could be processed to p52 upon XEDAR overexpression (Fig. 3B). Altogether, these findings indicate a role for NIK- and IKKα-mediated p100 phosphorylation in XEDAR-induced non-canonical NF-κB signaling.

3.4. XEDAR induces NIK stabilization

In the absence of an activating signal, cellular expression levels of NIK are kept low by its constitutive ubiquitination and proteasomal degradation, and by so doing, restrains non-canonical NF-κB signaling. It is believed that the physical association of NIK with TRAF2 and TRAF3 allows the TRAF2-cIAP E3 ubiquitin ligase complex to polyubiquitinate NIK. Recruitment of TRAF2 and TRAF3 to activated receptors such as LTβR initiates the polyubiquitination of TRAF proteins, and their subsequent proteasome-mediated degradation. The paradigm mentioned above posits that this receptor-mediated TRAF3 degradation allows NIK stabilization, and thus p100 activation [19].

To investigate whether a similar mechanism mediates XEDAR-induced non-canonical NF-κB signaling, we studied the effect of XEDAR overexpression on NIK expression levels. A clear increase in NIK levels was observed upon co-expression of XEDAR and NIK (Fig. 4A, lanes 1–3). Because pcDNA3, a plasmid used for the transient expression of NIK drives transcription via the CMV promoter, which can be activated by NF-κB, it was possible that the observed XEDAR-induced increase in NIK levels reflects XEDAR-induced NF-κB activation and increased NIK mRNA expression. Indeed, co-expression of the NF-κB inhibitor IκBα SR significantly reduced NIK expression, indicating a contribution of NF-κB mediated activation of the CMV promoter driving NIK expression. However, even under conditions in which NF-κB activation was inhibited by co-expression of IκBα SR we still observed higher amounts of NIK in the presence of XEDAR (Fig. 4A, lanes 4–6), indicating XEDAR-induced stabilization of NIK. To further support this, we also checked endogenous NIK expression levels, which is known to require prior enrichment by immunoprecipitation [20]. EDA-A2 stimulation of cells transfected with low amounts of XEDAR significantly increased the levels of endogenous NIK after 2 h stimulation (Fig. 4A, lower panel). This is consistent with XEDAR-induced NIK stabilization and our results obtained with NIK-transfected cells (Fig. 4A, upper panel). We subsequently addressed if XEDAR-induced NIK stabilization is associated with decreased TRAF3 expression, as has been observed for other receptors activating the non-canonical NF-κB pathway [4]. Indeed, overexpression of XEDAR was already sufficient to decrease the amounts of endogenous TRAF3 (Fig. 4B). These results are consistent with a model in which XEDAR-induced activation of the non-canonical NF-κB pathway involves the proteasomal degradation of TRAF3, leading to NIK stabilization and downstream activation of IKKα.

3.5. XEDAR-induced non-canonical NF-κB signaling is negatively regulated by TRAF3, cIAP1 and A20

Besides TRAF3, other critical components of the ubiquitin ligase complex that mediates NIK ubiquitination and destabilization are cIAP1/2 and TRAF2 [4]. It is believed that NIK is brought into close proximity to the cIAPs through a TRAF2-TRAF3 bridge where TRAF2 recruits c-IAP1/2 and TRAF3 binds to NIK. We therefore tested the effect of overexpression of TRAF2, TRAF3 and cIAP1 on XEDAR-induced p100 processing. As shown in Fig. 4C and D, TRAF3 and cIAP1 efficiently interfered with p100 processing, whereas TRAF2 had no effect. Most likely, the inability of TRAF2 to interfere with XEDAR-induced non-canonical NF-κB signaling reflects its dependence on endogenous TRAF3 expression, which is very low, to promote cIAP-dependent NIK ubiquitination.

It is likely that activation of non-canonical NF-κB signaling is subject to negative regulation by additional factors. We tested the ability of the deubiquitinase A20 (also known as TNFAIP3) to interfere with XEDAR-induced p100 processing. A20 is known as a key negative regulator of canonical NF-κB signaling in response to multiple stimuli (recently reviewed in Ref. [21]), but its effect on non-canonical NF-κB signaling is largely unknown. Recently, overexpression of A20 was shown to result in significant p100 processing and NIK accumulation in HEK293T cells [22]. Moreover, A20 was shown to promote efficient activation of the non-canonical pathway induced by stimulation of LTβR, Fn14, and RANK, but to

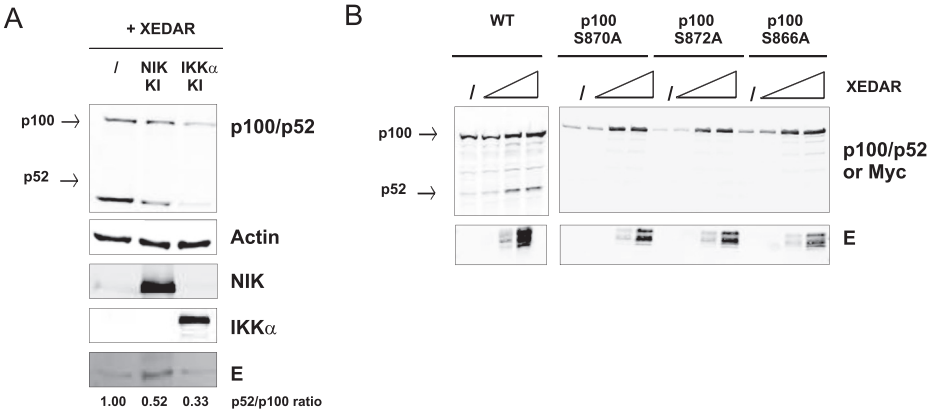

Fig. 3. NIK and IKKα-mediated phosphorylation of p100 is required for XEDAR-induced p100 processing. A. The kinase activities of NIK and IKKα are required for XEDAR induced p100 processing. HEK293T cells were transiently transfected with XEDAR together with kinase defective (KI) IKKα or NIK. After 24 h, cell lysates were analyzed by immunoblotting with anti-p100/p52. Expression of NIK, IKKα and XEDAR proteins was analyzed with an anti-NIK, anti-IKKα or anti-E-tag antibody, respectively. B. XEDAR-induced p100 processing depends on the known NIK and IKKα phosphorylation sites in p100. HEK293T cells were transiently transfected with XEDAR together with plasmids encoding wild type p100 or specific mutants as indicated. After 24 h, total lysates were made and analyzed by immunoblotting with anti-p100/p52 (wild type p100) or anti-Myc (p100 mutants) antibody. Expression of XEDAR was analyzed with an anti-E-tag antibody.

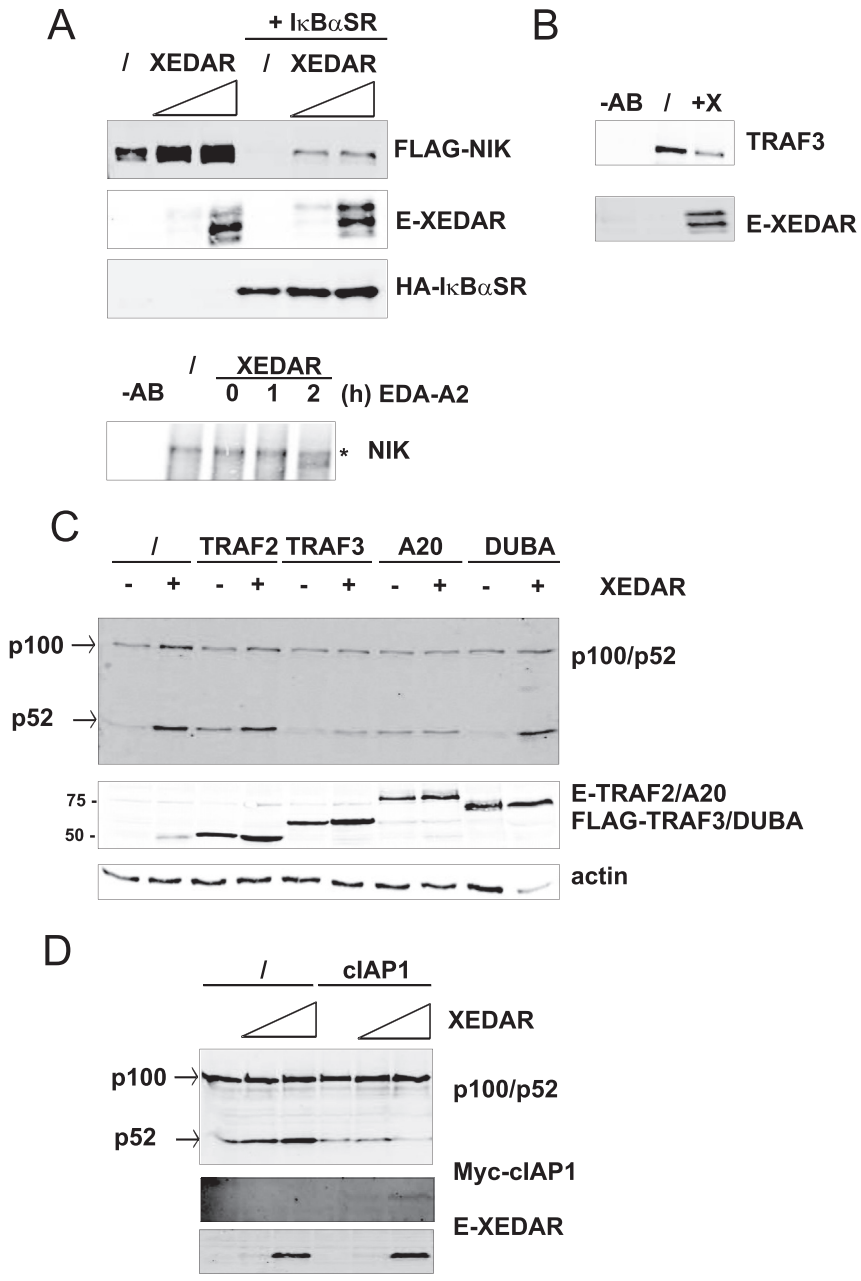

Fig. 4. XEDAR-induced non-canonical signaling is characterized by NIK stabilization and TRAF3 degradation and is subject to negative regulation by TRAF3, cIAP1 and A20. A. XEDAR induces NIK stabilization. Upper panel: HEK293T cells were transiently transfected with FLAG-NIK together with plasmids encoding E-XEDAR and HA-IκBα SR. After 24 h, total lysates were made and expression levels were analyzed by immunoblotting with antibodies against the FLAG, E or HA epitopes, respectively. Lower panel: HEK293T cells were transiently transfected with empty vector or XEDAR. 24 h later, cells were treated with EDA-A2 (10 ng/ml) for the indicated times. NIK levels were studied by immunoprecipitation with anti-NIK (lane 1, no antibody (AB) was added) and immunoblotted with anti-NIK. * indicates a non-specific band. B. XEDAR induces TRAF3 degradation. HEK293T cells were transiently transfected with E-XEDAR. After cell lysis, immunoprecipitation was performed with anti-TRAF3 (lane 1, no antibody was added) and immunoblotted with anti-TRAF3. Aliquots of total lysates were analyzed for expression of XEDAR by immunoblotting with anti-E tag. C,D. Negative regulators of the XEDAR-induced non-canonical NF-κB pathway. HEK293T cells were transiently transfected with E-XEDAR in combination with plasmids encoding FLAG-tagged TRAF2, TRAF3, and DUBA, E-tagged A20 or Myc-tagged cIAP1, as indicated. 24 h after transfection, total lysates were made and analyzed for p100 processing by immunoblotting with anti-p100/p52 antibodies. Expression of the tagged proteins was confirmed by immunoblotting with anti-E, anti-FLAG and anti-Myc antibodies.

be dispensable for activation upon CD40 stimulation [22]. Interestingly, this reported stimulatory effect of A20 on non-canonical NF-κB signaling was not associated with TRAF3 degradation, but due to direct binding to cIAP1, resulting in dissociation of the TRAF2/TRAF3 interaction, thereby preventing the ligase complex to destabilize NIK. Consistent with the above mentioned data, overexpression of A20 as such slightly induced p100 processing to p52 in our study (Fig. 4C). However, XEDAR expression was unable to further increase p100 processing in the presence of A20, suggesting that A20 negatively regulates XEDAR-induced non-canonical NF-κB signaling. Taken together, these results demonstrate that A20 regulates non-canonical NF-κB signaling in a receptor-dependent manner. We also tested the effect of the deubiquitinase DUBA (also known as OTUD5), which has been shown to selectively remove Lys63-linked polyubiquitin chains from TRAF3 upon TLR3 triggering [23]. However, in contrast to A20, DUBA overexpression was unable to prevent XEDAR-induced p100 processing (Fig 4C).

The mechanism by which A20 interferes with XEDAR-induced non-canonical NF-κB signaling remains to be investigated and will require the identification of XEDAR signaling proteins that undergo ubiquitination and that serve as substrates for A20. Alternatively, one cannot exclude a catalytic-independent function of A20. For example, since XEDAR-induced non-canonical NF-κB signaling requires the binding of different TRAF family members to XEDAR, and since A20 is known to interact with different TRAFs [24], it is tempting to speculate that A20 interferes with their binding to XEDAR.

In conclusion, the data presented here demonstrate the ability of EDA-A2 and XEDAR to stimulate non-canonical NF-κB signaling in HEK293T cells. This involves the signaling intermediates IKKα and NIK, and is negatively regulated by TRAF3, cIAP1 and A20. It will be interesting to study the ability of XEDAR to induce non-canonical NF-κB activation in other cell types. This will require the determination of cells that endogenously express XEDAR, which has so far been hampered by the unavailability of good XEDAR recognizing antibodies. XEDAR has been implicated in the regulation of ectodermal functions. For example, in humans a specific single nucleotide polymorphism in XEDAR is associated with androgenic alopecia [25]. Knockout of XEDAR in mice was reported to have no phenotypic effect [26], suggesting possible redundancy with other receptors. XEDAR was also identified as a p53 target gene, suggesting a possible tumor suppressor role of XEDAR [27]. The non-canonical NF-κB pathway forms a major arm of NF-κB signaling that mediates important biological functions, including lymphoid organogenesis, B-lymphocyte function, and cell growth and survival. Our data offer a platform for further studies on the role of XEDAR and non-canonical NF-κB signaling in normal and disease conditions.

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Transparency document

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