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Review

Expression, biological activities and mechanisms of action of A20 (TNFAIP3)

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ABSTRACT

A20 (also known as TNFAIP3) is a cytoplasmic protein that plays a key role in the negative regulation of inflammation and immunity. Polymorphisms in the *A20* gene locus have been identified as risk alleles for multiple human autoimmune diseases, and A20 has also been proposed to function as a tumor suppressor in several human B-cell lymphomas. A20 expression is strongly induced by multiple stimuli, including the proinflammatory cytokines TNF and IL-1, and microbial products that trigger pathogen recognition receptors, such as Toll-like receptors. A20 functions in a negative feedback loop, which mediates its inhibitory functions by downregulating key proinflammatory signaling pathways, including those controlling NF-κB- and IRF3-dependent gene expression. Activation of these transcription factors is controlled by both K48- and K63- polyubiquitination of upstream signaling proteins, respectively triggering proteasome-mediated degradation or interaction with other signaling proteins. A20 turns off NF-κB and IRF3 activation by modulating both types of ubiquitination. Induction of K48-polyubiquitination by A20 involves its C-terminal zinc-finger ubiquitin-binding domain, which may promote interaction with E3 ligases, such as Itch and RNF11 that are involved in mediating A20 inhibitory functions. A20 is thought to promote de-ubiquitination of K63-polyubiquitin chains either directly, due to its N-terminal deubiquitinase domain, or by disrupting the interaction between E3 and E2 enzymes that catalyze K63-polyubiquitination. A20 is subject to different mechanisms of regulation, including phosphorylation, proteolytic processing, and association with ubiquitin binding proteins. Here we review the expression and biological activities of A20, as well as the underlying molecular mechanisms.

Contents

1. Introduction

In vertebrates, the innate and adaptive immune systems protect against invading pathogens and tissue damage. Innate immunity forms the first line of defence and is triggered by recognition of pathogen-associated molecular patterns (PAMPs) by germline-encoded receptors on phagocytic cells, such as macrophages, neutrophils and dendritic cells. Recognition of PAMPs involves a variety of receptors, including Toll-like receptors (TLRs), Nod-like receptors (NLRs) and RIG-I-like receptors (RLRs), which recognize their ligands either at the cell surface or intracellularly [1]. Upon activation of these receptors, multiple signaling cascades are

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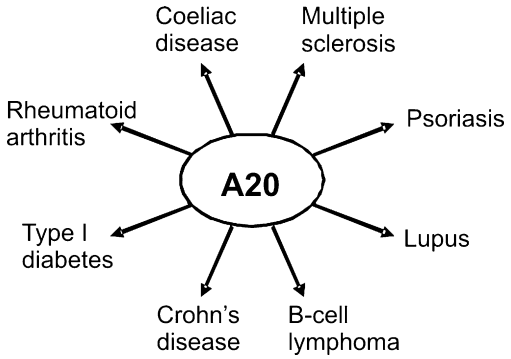

Fig. 1. A20 is a susceptibility gene for multiple diseases.

triggered, leading to the activation of nuclear factor-κB (NF-κB), interferon regulatory factor 3 (IRF3) and activator protein 1 (AP-1) transcription factors. Together, these transcription factors induce expression of genes which mediate the immediate effector functions of innate immune cells, such as iNOS and tissue destructive enzymes, together with inflammatory cytokines and chemokines, which are important in attracting T cells to sites of infection to initiate the adaptive immune response.

Activation of innate and adaptive immune responses is tightly regulated, as insufficient activation could result in defective clearance of pathogens, while excessive activation might lead to lethal systemic inflammation or autoimmunity. A20 is an important negative regulator of innate and adaptive immunity, both after acute infection and in immune homeostasis. The importance of A20 in limiting inflammation is underscored by the association of polymorphisms in the A20 genomic region with multiple human autoimmune and inflammatory diseases, including Crohn's disease, rheumatoid arthritis, systemic lupus erythematosus, psoriasis and type 1 diabetes (Fig. 1). Furthermore, A20 also functions as a tumor suppressor in several B cell lymphomas. The role of A20 as a disease associated gene has recently been reviewed in detail elsewhere [2,3]. Here we summarize what is known about the expression, biological activities, and mechanism of action of A20.

2. Expression of A20

A20 was initially identified as a primary response gene following stimulation of human umbilical vein endothelial cells (HUVEC) with TNF, IL-1 or LPS [4]. Subsequently, A20 was also found to be transcriptionally upregulated in other cell types, by many other agonists (see Table 1). In contrast, human immature $CD4^+CD8^+$ double positive thymocytes and single positive thymocytes constitutively express significant levels of A20, but upon stimulation with TPA/ionomycin, A20 expression declines in the mature single-positive cells. Similarly, A20 is expressed in resting peripheral T-cells, and is down-regulated after activation [5].

Dysregulation of A20 expression in certain transformed cells suggests that A20 may play a role in the pathogenesis of cancer. A20 mRNA is upregulated in undifferentiated nasopharyngeal carcinoma and poorly differentiated head and neck squamous cell carcinomas (SCCs) of the skin, suggesting that A20 has a part in the pathogenesis of these epithelial malignancies. In contrast, no A20 mRNA is detected in normal samples of squamous epithelial tissues or in well-differentiated SCCs [6]. A20 expression in estrogen receptor (ER) and progesterone receptor (PR) negative breast tumour cells *in vivo* has been associated with poor prognosis. A20 mRNA levels are significantly higher in ER-negative breast cancer cell lines and tamoxifen resistant ER-positive tumours. Furthermore, estrogen stimulation of the MVLM cell line, an ER-positive human breast carcinoma, downregulates A20 mRNA expression [7]. A20 expression is also increased in inflammatory breast cancer, a frequently hormone receptor negative progressive form of cancer [8]. Together, these data demonstrate that a high expression of A20 is often linked with poor prognosis of epithelial malignancies. On the other hand, a large number of B-cell lymphomas contain mutations in A20 that result in loss of A20 protein expression, indicative for a tumor suppressor role for A20 [9], and suggesting that the role of A20 in tumorigenesis might be cell type dependent.

In mice, constitutive *A20* mRNA expression can be detected in epithelial cells involved in early hair follicle development, but the highest levels of *A20* mRNA are seen in lymphoid tissues, like thymus, gut-associated lymphoid aggregates (containing B- and T-cells) and spleen [5]. Moreover, TNF injection in mice results in a substantial upregulation of *A20* mRNA in liver, kidney, spleen, thymus, lung, colon and lymph nodes [10].

The human *A20* gene is located on chromosome 6q23 [11], while the murine *A20* gene is mapped to chromosome 10 at 13 cM [5,12]. Human *A20* mRNA is 4 kb long, containing an open reading frame of 2370 nucleotides, encoding a protein of 790 amino acids with a molecular weight of 90 kDa [13]. The 3′-untranslated region contains four copies of the sequence ATTTA, an mRNA destabilizing motif that is frequently found in the 3′-UTR of early response inflammatory mediators. A20 mRNA is superinduced by cycloheximide [4], suggesting a role for protein synthesis in the destabilization of A20 mRNA. Reporter assays indicate that transcriptional activation of the *A20* gene is mediated by two NF-κB binding sites in its promoter [14]. Consistently, *A20* expression is reduced in cells genetically deficient in NF-κB activation [15]. Furthermore, A20 negatively regulates its own expression as a consequence of its NF-κB inhibitory activity [14], resulting in *A20* mRNA only being transiently induced following TNF stimulation [4]. The rapid induction of the *A20* gene upon TNF stimulation has been suggested to involve the constitutive association of the general transcription apparatus and co-activators, such as CBP and p300, with the *A20* promoter, mediated by the transcription factor Sp-1 [16]. While Sp-1 activity is necessary for the efficient transcription initiation, NF-κB binding to the promoter enhances the re-initiation rate, resulting in multiple transcription rounds and augmenting expression levels. The basal activity of the *A20* gene is repressed at the elongation step by DRB sensitivity-inducing factor (DSIF), whose activity is controlled by a negative upstream promoter element called ELIE (elongation-inhibitory element) [17]. Remarkably, following stimulation, inhibition of the *A20* promoter by DSIF persists, but it is now regulated by NF-κB rather than ELIE. Recently, an E-box, overlapping with ELIE, in the *A20* promoter was demonstrated to bind upstream stimulatory factor (USF)1 [18]. USF1 mediates DSIF inhibition and recruitment to the *A20* promoter under basal conditions. Once NF-κB is induced, the E-box is no longer involved in DSIF inhibition. Consistent with that, USF1 is displaced from the promoter by NF-κB (Fig. 2).

3. A20 structure

The amino acid sequence of A20 is highly conserved (more than 90% identical when compared to human A20) in various mammalian species, and clear orthologs are present in Zebrafish and Xenopus (50% identical to human A20, but up to 90% in the N-terminal A20 domain). In contrast, no such clear orthologs can be found in Drosophila and C. elegans [19]. Homology comparisons have revealed the presence of an ovarian tumor (OTU) domain in the N-terminal part of A20 (Fig. 3) [20], suggesting that A20 functions as a deubiquitinase, which has been confirmed biochemically [21]. The crystal structure of the OTU-domain shows ten β-strands and ten α-helices, which are arranged to form an expansive surface on which ubiquitin binding, interaction with substrates and catalysis can take place [22,23]. The catalytic site is composed of a catalytic triad (Cys103, His256, Asp70), with a highly conserved surface patch,

Table 1
Expression pattern of A20.

Stimulus	Cell type	Refs.
TNF	Osteosarcoma cell line U2OS	[90]
	Vascular smooth muscle cells (VSMC)	[85]
	Jurkat T-cells	[15]
	Human microvascular endothelial cells (HMEC-1)	[82]
	Ventricular myocytes	[91]
	Myotubes	[92]
	Osteoblastic MC3T3-EI cell line	[93]
	Primary human hepatocytes, hepatocellular carcinoma cell line HepG2	[94]
	MCF-7 breast carcinoma cell line	[33]
	β cells (primary islets, Min6)	[95]
IL-1	β cells (primary islets, Min6)	[95]
LPS	Bone marrow-derived macrophages (BMDMs)	[51]
	Primary human airway epithelial cells	[96]
	HMEC-1 cells	[82]
	Bone marrow-derived dendritic cells (BMDCs)	[97]
	Freshly isolated human blood monocytes	[98]
	Enterocytes (IEC-6, IEC-18, RIE-1, SW480, HCT-15)	[98,99]
CpG DNA	Enterocytes (IEC-18)	[99]
Flagellin	Enterocytes (HCT-15, HT-29, mouse primary cells, Colon-26)	[98]
CD40	B-cells	[100]
	Fibroblast cell line SV80	[101]
TPA	Jurkat T-cells, U937 promonocytic cells	[102]
	Freshly isolated human blood monocytes	[103]
TRAIL	MCF-7	[104]
Angiotensin II	Human adrenocortical cells	[105]
Peptidoglycan	Primary human airway epithelial cells	[96]
H_2O_2	HeLa cells	[43]
Serum from patients with burn injuries	Human umbilical vein endothelial cells (HUVEC)	[50]
TNF-RII	Murine CT6 cells	[58]
Androgen	LNCaP prostate cancer cells	[106]
CD3	Do-11.10 T cell hybridomas, $CD4^+$ and $CD8^+$ T-cells	[39]
Vitamin D_3	Increase of expression:	[107,108]
	* rat insulin-producing β-cells (RINm5F)	
	* human islet cells	
	* liver allografts	
	Decrease of expression:	[106]
	* LNCaP cells	
Zinc depletion	Diminished LPS- and TPA-induced expression in promyelocytic leukemia HL-60 cells	[109]
ER stress	Mesangial cells, NRK-52E cells	[110]
Epstein-Barr virus latent membrane protein LMP1	B-cells	[111]
	Human epithelial cells	[112]
Human T-cell leukemia virus type I oncoprotein Tax	Jurkat T cells	[102]
Human cytomegalovirus	Human peripheral blood monocytes	[113]
Influenza virus	Human bronchial epithelial cells, lung homogenates from mice	[114]
Sendai virus	U937	[54]
Measles virus P protein	Monocytes (THP1, U937)	[115]
Hepatitis C virus core protein	HepG2	[116]
Helicobacter pylori	Human gastric cancer cells	[117]
Uropathogenic Escherichia coli	Mice bladder tissues	[118]
VLA-4 stimulation	Monocytes	[119]

forming the ubiquitin-binding site and a separate region for binding target proteins. Cezanne and TRABID are two other OTU-domain containing deubiquitinases that show considerable similarity with A20 within their OTU-domain, and have therefore been proposed as A20-like proteins [24,25].

The C-terminal domain of A20 contains seven zinc finger (ZnF) structures, of which 6 have the sequence $CX_4CX_{11}CX_2C$ and one is similar to the sequence $CX_2CX_{11}CX_2C$ (Fig. 3) [13]. The human and mouse A20 ZnFs are completely identical, except for a single substitution of methionine for valine in ZnF4 [5]. It has been proposed that ZnF4 confers intrinsic ubiquitin ligase activity to A20 (see Section 4.3). The C-terminal ZnF containing domain is also required for homo-oligomerization of A20, although it is not clear whether this is important for A20 function [13]. The A20-related protein Cezanne only contains a single A20-like zinc finger in its C-terminus, which is most similar to ZnF4 and ZnF7 of A20 [25]. Also the A20-related protein TRABID contains three ZnFs at its N-terminus, but these are unrelated to those of A20. Finally, A20-like ZnFs have also been described in other proteins such as ZNF216 and Rabex-5 [26,27].

Between ZnF3 and ZnF4 of A20, there is a perfect 14-3-3 binding sequence (RSKSDP), which is conserved between human and mouse (Fig. 3) [28,29]. Consistently, A20 specifically interacts with the η and ζ isoforms of 14-3-3, as mutations in the consensus 14-3-3 binding site abolish these interactions [28]. Interaction of A20 with 14-3-3 alters the localization of A20 from a punctuate cytoplasmic staining to a more diffuse cytoplasmic pattern, which is also associated with decreased amounts of A20 in the insoluble cell fraction [29]. Mutations in the 14-3-3 binding domain of A20 lead to proteolysis of A20 in MCF-7 cells [30], suggesting that the

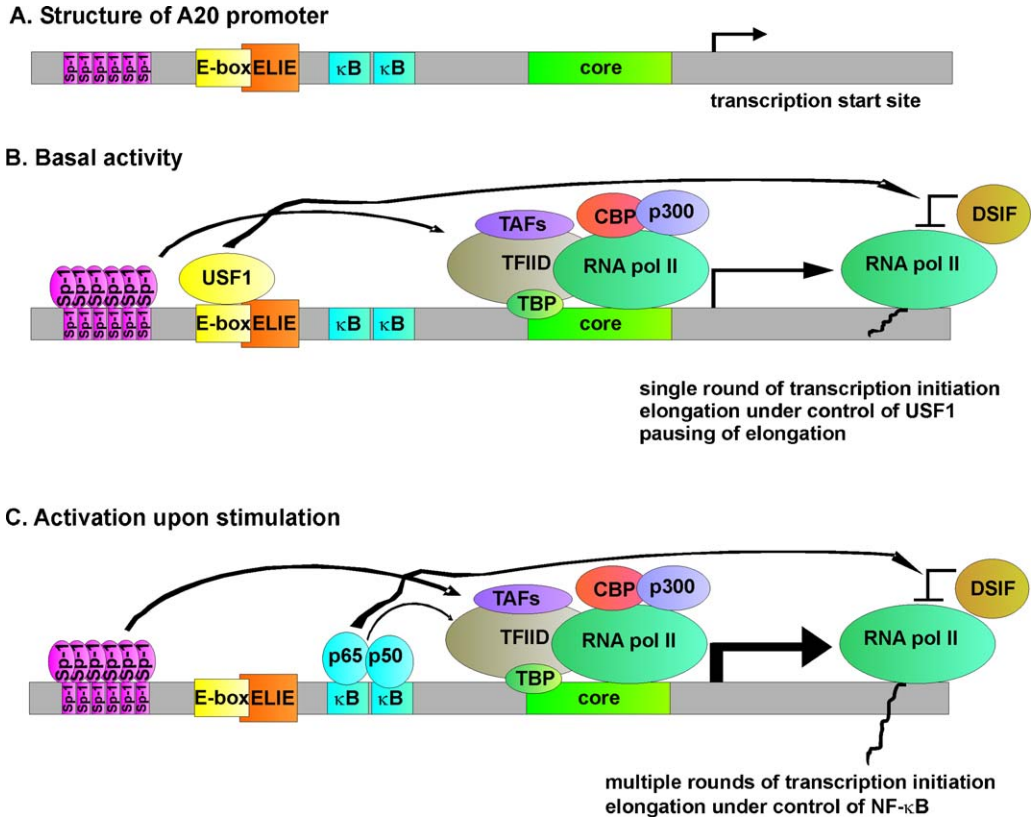

Fig. 2. Structure and activation of the A20 promoter. (A) Structure of A20 promoter. A20 contains upstream of the core promoter two binding sites for NF-κB, six Sp-1 sites, and an E-box overlapping the ELIE site. (B) Under basal conditions, the general transcription apparatus (composed of RNA polymerase II, TFIID, TAFs, TBP and others), together with the co-activators CBP and p300, is constitutively associated with the core promoter. Together with Sp-1, this allows a single round of transcription initiation. In addition, A20 mRNA elongation is inhibited by DSIF, whose activity is controlled by the E-box binding protein USF1. (C) Upon stimulation, NF-κB is activated and binds to the κB sites in the A20 promoter, leading to re-initiation and multiple rounds of transcription. In addition, NF-κB displaces USF1 from the promoter and takes over the control of DSIF activity, permitting mRNA elongation by RNA polymerase II.

binding to 14-3-3 protects A20 from degradation. Although A20 is mostly localized in the cytosol, a fraction of A20 can associate with a lysosome-interacting compartment in a manner that requires its carboxy terminal ZnFs [31]. This lysosome-associated A20 has been shown to target the A20-associated signaling molecule TRAF2 to the lysosomes for degradation [32].

4. Biological activities of A20

4.1. Cytoprotective effects of A20

The first function described for A20 was its cytoprotective effect upon TNF stimulation of cells, based on the effect of

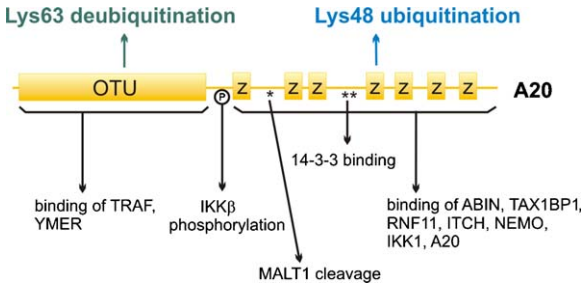

Fig. 3. Domain structure of A20. A20 consists of an N-terminal OTU domain and 7 C-terminal zinc fingers (z), mediating respectively K63 deubiquitination and K48-polyubiquitination. Regions of phosphorylation, proteolytic cleavage and binding of other proteins are indicated.

A20 overexpression [33]. This was later confirmed genetically as *A20*$^{-/-}$ murine embryonic fibroblasts and thymocytes were found to be more sensitive to TNF-induced cell death than wild type (WT) cells [10]. However, the anti-apoptotic function of A20 is not a general feature, as A20 only protects some cell types from specific death inducing agents. For example, overexpression of A20 protects MCF7S1 and WEHI-S cells from TNF-mediated apoptosis, but not from killing by anti-Fas, activated monocytes, serum starvation or H_2O_2 [34]. In contrast, A20 protects endothelial cells against apoptosis induced by TNF and Fas triggering [35], whereas in A549, HepG2 and HeLa cells A20 is not able to block TNF-induced apoptosis [36,37]. Induction of A20 after viral infection has also been suggested as a mechanism to protect infected cells from p53-induced apoptosis [38], and A20 downregulation may be important in activation-induced cell death (AICD) in T-cells [39]. Table 2 summarizes the effects of A20 on cell survival in different cell types. It should be mentioned that our knowledge of the cytoprotective effects of A20 and the underlying mechanisms (see below) is mostly based on studies using A20 overexpression in cell lines, implicating the need to confirm their physiological relevance using A20-deficient primary cells.

The underlying mechanism by which A20 inhibits apoptosis is still largely unclear. Overexpression experiments indicate that the C-terminal ZnF containing domain of A20 is necessary and sufficient for its anti-apoptotic activity following TNF stimulation, but the 14-3-3 binding site is dispensable [30,36]. In Jurkat T-cells, ectopic expression of A20 inhibits TNF-induced apoptosis by preventing recruitment of RIP1 and TRADD to the TNF-RI complex,

Table 2
Biological activities of A20.

Function	Stimulus	Cells	Evidence	Mechanisms	Refs.
Anti-apoptotic	TNF	Thymocytes, MCF7S1, WEHI-S, endothelial cells, β-cells, Jurkat, rat primary hippocampal neurons, NIH3T3, the osteoblastic MC3T3-E1, SV80, primary hepatocytes, NIH3T3, primary glioma stem cells	knockout, overexpression, RNAi	* prevention of recruitment of RIP1 and TRADD to TNF-RI * inhibition of p53 * inhibition of Bax expression	[10,15,33, 36,38,94, 96,101, 120–123]
	Fas	Endothelial cells	overexpression		[35]
	TRAIL	Lung carcinoma (H460)	overexpression	*Caspase 8 deubiquitination	[40]
	Natural killer cells	Endothelial cells	overexpression		[35]
	Serum deprivation	B-cells	overexpression		[100]
	Serum from patients with burn injuries	Human umbilical vein endothelial cells (HUVEC)	overexpression		[50]
	LPS	Human microvascular endothelial cells (HMEC-1)	overexpression		[82]
	CD40	Human coronary artery endothelial cells (HCAEC)	overexpression		[49]
	AICD	Do-11.10 T cell hybridomas	overexpression		[39]
	etoposide	Lymphoblastoid cell lines	RNAi		[124]
	H_2O_2	Lymphoblastoid cell lines	RNAi		[124]
Anti-necrosis	TNF	L929	overexpression	* inhibition of phospholipases; production of reactive oxygen species	[41,43]
	Complement	Endothelial cells	overexpression		[35]
	Hypoxia-reoxygenation	Mouse hepatocyes (NMuLi)	overexpression	* increase in PPARα expression	[125]
	H_2O_2	Mouse hepatocyes (NMuLi)	overexpression	* increase in PPARα expression	[125]
Pro-apoptotic	Fas-and cytokine mediated	Vascular smooth muscle cells (VSMC)	overexpression	*NF-κB inhibition	[43,126]
NF-κB inhibition	TNF	MCF7S1, bovine aortic endothelial cells (BAEC), HEK293T, MEF, HeLa	overexpression, knockout, RNAi	* ubiquitin editing of RIP1, leading to its degradation * deubiquitination of NEMO * inhibition of TRAF/cIAP1 E3 ligase activity by antagonizing the interaction with E2 enzymes * induction of proteasomal degradation of E2 enzymes * degradation of TRAF2 via the lysosomes	[10,34,44, 58,62,65,96] [69] [32]
	IL-1	MCF7S1, HEK293T, human airway epithelial cells,	overexpression, knockout	* deubiquitination of TRAF6	[34,44,51, 56,58,96]
	LPS	HEK293, BAEC, macrophages		* inhibition of TRAF6 E3 ligase activity by antagonizing the interaction with E2 enzymes * induction of proteasomal degradation of E2 enzymes	[69]
	H_2O_2	HeLa, endothelial cells	overexpression, RNAi		[43,47]
	TPA	BAEC, HEK293T	overexpression		[44] [71]
	anti-CD3	Jurkat	RNAi	*MALT1 deubiquitination	[70]
	API-MALT1	HEK293T	overexpression		[71]
	RIG-I	HEK293	overexpression		[53]
	Helicobacter pylori	Human gastric cancer cells	overexpression		[117]
	Influenza virus	Human bronchial epithelial cells	overexpression		[114]
	Peptidoglycan	Human airway epithelial cells	overexpression		[96]
	Serum from patients with burn wounds	HUVEC	overexpression		[50]
	CD40	HEK293T	overexpression		[58]
	TNF-RII	HEK293T	overexpression		[58]
	NOD1/NOD2	HEK293T	overexpression	* deubiquitination of RIP2	[64]
	LMP1	Human keratinocytes, HEK293	overexpression		[45,127]
AP-1 inhibition	TNF	MCF7S1	overexpression		[34]
	IL-1	MCF7S1	overexpression		[34]
	LMP1	HEK293	overexpression		[127]

Table 2 (*Continued*)

Function	Stimulus	Cells	Evidence	Mechanisms	Refs.
	TLR-4	HEK293	overexpression		[56]
IRF3 inhibition	RIG-I	HEK293	overexpression		[53]
	TRIF	HEK293	overexpression	* reduction of TRIF expression	[53]
	Sendai virus	HEK293	overexpression, RNAi		[53,54]
	Poly(I:C)	HEK293/TLR3, HeLa, THP-1	overexpression, RNAi		[54,55]
	Newcastle disease virus	HEK293/TLR-3	overexpression, RNAi		[55]
	IKKε/TBK1 VSV	HEK293	overexpression	*Inhibition of IKKε/TBK1 ubiquitination by disrupting their interaction with TRAF3 E3 ligase	[75]
Skin differentiation			knockout mice	* impaired NF-κB activation * interaction with IKK1	[5,10,57]
Cell cycle arrest		VSMC	overexpression	* arrest in G_0/G_1 by inhibition of PI3K/Akt/GSK3β signaling * upregulation of p53, $p21^{waf1}$ and $p27^{kip}$	[84,85]
Mitogenic in the liver			adenoviral gene delivery in mice		[94]
Neovascularisation		HUVEC	RNAi		[86]

which mediate the recruitment of FADD and the initiator procaspase 8, leading to its autoactivation by proteolytic cleavage [15]. In endothelial cells, however, A20 overexpression was shown to restrict not only TNF- but also Fas-induced apoptosis, associated with the inhibition of proteolytic cleavage of apical caspases 8 and 2, executioner caspases 3 and 6, Bid cleavage, and release of cytochrome c, thus preserving mitochondrion integrity [35]. Inhibition of both TNF-RI- and Fas-mediated apoptosis suggests that A20 interferes at a common step in their downstream signaling pathways. The two death receptors differ only in their use of proximal signaling molecules. TNF-RI requires the adapters TRADD and RIP1 to transduce signals to FADD, whereas Fas interacts directly with FADD and activates caspase 8. Inhibition of both of these death pathways in endothelial cells supports an effect of A20 at the level of FADD/caspase 8. Recently, A20 was shown to reverse death receptor-induced polyubiquitination of caspase 8 [40]. As the latter is necessary for caspase 8 aggregation, leading to its full activation and processing, it is tempting to speculate that A20 mediates its anti-apoptotic activities by deubiquitinating caspase 8.

A20 not only inhibits TNF-induced apoptosis, but also TNF-induced necrosis as shown by overexpression of A20 in the murine fibrosarcoma L929 cell line [41], which is associated with reduced activation of phospholipases and reduced induction of reactive oxygen species. Similarly, A20 overexpression protects endothelial cells from complement-mediated, but not heat-induced necrosis [35]. Recently, A20 was shown to inhibit both the caspase 8 dependent and independent cell death pathway in macrophages stimulated with TNF [42]. Overexpression of A20 in these cells protected against the loss of lysosomal integrity and cathepsin B release (caspase 8 independent and typical for necrosis) and DNA fragmentation (RIP1 and caspase 8 dependent and typical for apoptosis). The mechanism for the anti-necrotic action of A20 is still unknown.

In contrast to its cytoprotective effects, A20 has also been reported to sensitize vascular smooth muscle cells to cytokine- and Fas-mediated apoptosis [35]. In addition, cells expressing high levels of A20 were shown to be more sensitive to oxidative stress-mediated cell death than cells with low A20 levels [43]. The reason for these paradoxical effects is still unclear, but might reflect the delicate balance between a direct anti-apoptotic effect of A20 and an indirect apoptosis-sensitizing effect due to its NF-κB inhibitory function.

4.2. Negative regulation of inflammation by A20

Early experiments in cell lines suggested an anti-inflammatory role for A20, since overexpressed A20 was found to substantially inhibit NF-κB activation in response to several different stimuli (see Table 2). Consistently, inhibition of NF-κB activation by A20 expression decreases expression of several NF-κB target genes, including E-selectin, ICAM-1, IL-8, IκBα, tissue factor, VCAM-1, IL-6 and GM-CSF [44–50]. A physiological function of A20 in the negative regulation of inflammation was confirmed by the phenotype of A20 knockout mice [10]. $A20^{-/-}$ mice die shortly after birth by severe inflammation and tissue damage in multiple organs, including liver, kidneys, intestine, joints and bone marrow. $A20^{-/-}$ $RAG1^{-/-}$ mice, which are devoid of T- and B-cells, display the same phenotype, indicating that A20 regulates innate immune homeostasis independent of adaptive immune cells [51]. In accordance with A20 overexpression experiments, analysis of MEFs from $A20^{-/-}$ mice demonstrates a role for A20 in regulating NF-κB activation [10]. TNF stimulation of A20-deficient MEFs results in prolonged IKK activation compared to WT cells. This causes sustained phosphorylation and degradation of IκBα, and consequent increased NF-κB activity in A20-deficient cells. The finding that A20/TNF-RI and A20/TNF double knockout mice have a similar phenotype as the A20 single knockout mice, suggest a critical role for A20 in the regulation of TNF-independent pro-inflammatory signals and the maintenance of immune homeostasis [51]. LPS activation of IKK is prolonged in macrophages generated from A20/TNF double knockout mice, indicating that A20 negatively regulates TLR4 activation of NF-κB, independently of TNF. Indeed, recent experiments have suggested that the inflammatory phenotype of $A20^{-/-}$ mice is due to excessive signaling from MyD88-dependent TLRs (such as TLR3, TLR4 and TLR9); unlike, $A20^{-/-}$ mice, $A20^{-/-}MyD88^{-/-}$ mice do not die prematurely or exhibit severe cachexia and have reduced

inflammation in their spleens [52]. Using broad spectrum antibiotics treatment it was further shown that commensal intestinal bacteria drive these constitutive TLR signals in the absence of A20. It should be noted that *A20*$^{-/-}$ macrophages also express more TNF, IL-6 and NO after triggering of TLR3, which is MyD88-independent, suggesting that A20 also interferes with the TRIF-dependent TLR signaling axis [51].

Clear inhibitory effects of A20 have also been observed on IRF3 activation. For example, A20 overexpression strongly inhibits RIG-I mediated activation of IRF3 [53]. A20 overexpression has also been found to inhibit ISRE- and IFN-β promoter-dependent luciferase activity upon poly(I:C) stimulation of TLR3 [54], as well as Newcastle disease virus (NDV)-induced IRF3 activation in HEK293 cells [55]. Consistent with these data, A20 knock down in HEK293T, HeLa and THP-1 cells enhances ISRE-dependent transcription after poly(I:C) stimulation, infection with NDV [55] or Sendai virus [53]. Co-transfection data suggest that A20 inhibits TLR3-mediated IRF3 activation upstream or at the level of TBK1/IKKε kinases, possibly involving direct interaction of A20 with TBK1, IKKε and TRIF. However, analysis of *A20*$^{-/-}$ mice suggest that the effects of A20 on expression of IRF3-regulated genes may be indirect. Irradiated mice reconstituted with hematopoietic stem cells derived from *A20*$^{-/-}$ *MyD88*$^{-/-}$ mice have higher levels of IFN-β and MCP-1, two TRIF-dependent pro-inflammatory cytokines, after LPS injection [52]. However, in BMDMs generated from these mice, the kinetics of IRF3 phosphorylation after LPS stimulation are comparable to those in wild-type cells, while IκBα phosphorylation is sustained due to prolonged IKK activation. These results suggest that A20 may restrict TRIF-dependent signaling to IFN-β by regulating NF-κB activation and not IRF3. A20 may control common signaling proteins involved in MyD88- and TRIF-dependent signaling to NF-κB, but not implicated in IRF3 activation. Possible candidates are TRAF6 and RIP1 (see Section 4.3).

A20 overexpression has additionally been reported to inhibit AP-1 activation following TNF, IL-1 and LPS stimulation [34,56]. However, AP-1 inhibition by A20 has not always been detected [48,57] and may be cell type- or stimulus-specific. The AP-1 inhibitory effect can be explained by the inhibition of JNK activation by A20 [45], although this has not been detected in all cells [47]. Results obtained from A20-deficient MEF cells support a role for A20 in the negative regulation of TNF-induced JNK activation [10].

4.3. Mechanisms involved in the NF-κB inhibitory effect of A20

As discussed in Section 4.2, analysis of A20-deficient cells has indicated that A20 negatively regulates TNF and TLR4 activation of IKK. Consistent with these data, A20 has been shown to inhibit NF-κB activation by co-expressed signaling intermediates which function upstream of the IKK complex, such as TAK1, TRAF2/6, TRADD and RIP1 [37,48,57,58], but does not affect NF-κB activation by overexpressed RelA [37,48]. However, contradictory data have been generated on the effect of A20 on nuclear translocation and DNA-binding of NF-κB. For example, overexpression of A20 in HEK293T and L929sA cells inhibits TNF-induced NF-κB dependent luciferase expression without affecting nuclear translocation and DNA-binding of NF-κB, suggesting that A20 specifically interferes with an NF-κB transactivation signal [48,57]. It will clearly be important to obtain complementary evidence for A20 regulation of NF-κB transactivation using A20-deficient cells.

Co-expression experiments have demonstrated that several signaling intermediates upstream of the IKK complex can bind to A20 (see Table 3). For example, A20 interacts with TRAF1, TRAF2 and TRAF6, which was shown to be mediated by its N-terminal domain in the case of TRAF1 and TRAF2 [58,59]. A20 has also been shown to interact with IKK1 and NEMO via its C-terminal ZnF domain [60,61], but not with IKK2 [57]. In accordance with these data, endogenous A20 is recruited to the TNF-RI signaling complex after TNF stimulation [61]. The ability of A20 to bind to proximal components of the TNF-RI signaling pathway to NF-κB is consistent with data indicating that A20 regulates IKK activation after TNF stimulation.

Wertz et al. proposed that A20 contains two ubiquitin-editing domains, an N-terminal deubiquitinating (DUB) domain of the OTU family and a C-terminal ubiquitin ligase domain [62]. The N-terminal domain was shown to deubiquitinate K63-polyubiquitinated RIP1, an essential mediator of the proximal TNF-RI signaling complex. The C-terminal ZnF containing domain functions as an ubiquitin ligase that catalyzes the addition of K48-polyubiquitin chains to RIP1, thereby targeting RIP1 for proteasomal degradation [62,63] (Fig. 4). A20 mutated at the OTU-domain catalytic cysteine residue is defective in adding K48-polyubiquitin chains to RIP1, suggesting that RIP1 K63 deubiquitination is a prerequisite for A20-mediated RIP1 K48-polyubiquitination and subsequent degradation. The catalytic cysteine is also necessary for deubiquitination of K63-polyubiquitinated TRAF6 by A20 (Fig. 4) and inhibition of NF-κB activation after LPS stimulation [51]. Furthermore, stimulation of NOD2 with MDP leads to K63-polyubiquitination of RIP2, which can be inhibited by A20 depending on its OTU domain [64]. A20 has also been reported to remove K63-polyubiquitin chains from NEMO in TNF-stimulated cells. However, the role of NEMO ubiquitination in NF-κB activation is unclear [65,66]. As discussed earlier, *in vitro* the N-terminal domain of A20 has been shown to hydrolyse both K48- and K63-polyubiquitin [21], and hydrolysis of K48-polyubiquitin chains was later shown to be much more efficient than K63-polyubiquitin chains [22,23]. This suggests that other factors such as the polyubiquitinated protein itself or possibly other A20-binding proteins might determine the DUB specificity of A20 for K63-polyubiquitinated substrates in intact cells.

Reconstitution of *A20*$^{-/-}$ MEF cells with the OTU catalytic cysteine mutant of A20 is much less effective in down-regulating TNF-induced NF-κB activity than wild type A20 [62], suggesting that DUB activity is essential for A20's inhibitory function. However, several studies showed that the OTU domain is not important for the NF-κB or IRF3 inhibitory activity of over-expressed A20. For example, overexpression of the C-terminal domain or the OTU catalytic cysteine mutant of A20 still inhibits TNF-, IL-1- and Pam3Cys-induced NF-κB activation, as well as poly-(I:C)- and NDV-induced IRF3 activation, as potently as the wild type molecule [21,30,48,55,58]. The physiological relevance of such overexpression experiments remain to be demonstrated.

ZnF4 is essential for retrovirally expressed A20 to trigger RIP1 proteolysis in *A20*$^{-/-}$ MEFs and it has been suggested that ZnF4 confers ubiquitin ligase activity to A20 [62]. However, it is unclear whether A20 has intrinsic E3 ligase activity or if this activity is mediated by an associated protein such as Itch and RNF11, which are two A20-binding E3 ubiquitin ligases that have been reported to be essential for A20's NF-κB inhibitory function [67,68]. It is also evident that ZnF4 is not always required for A20 to inhibit NF-κB. For example, the inhibitory effect of over-expressed A20 on RIG-I- and virus-induced NF-κB and IRF3 activation was found to involve ZnF7 and not ZnF4 [53]. Likewise, ZnF7 contributes to the inhibition of TNF-induced NF-κB activation by overexpressed A20 [37], as well as to the localization of A20 to lysosome-associated compartments [31]. Furthermore, oligomerization of ZnF7 as such inhibits NF-κB activation [37]. Klinkenberg et al. demonstrated that there is redundancy between the ZnFs of A20 for inhibition of TNF-induced NF-κB activation, with 4 ZnFs being sufficient for inhibition [60]. However, it should be noted that either ZnF4 or ZnF7 were still present in inhibitory A20 mutants.

Table 3
A20-interacting proteins.

Interacting protein	Evidence	Interacting domain in A20	Interacting domain in partner protein	Ref
A20	Y2H	C-terminal ZnF-domain (AA 373-790, human)		[128]
14-3-3	Y2H, in vitro binding assay, COIP (overexpression, semi-endogenous)	RSKSDP sequence (human and murine) between ZnF3 and ZnF4, with the second serine being phosphorylated		[28,29]
NEMO	Y2H, COIP (overexpression, semi-endogenous upon overexpression of TNF-RI)	C-terminal ZnF-domain (AA 387-790, human) (AA 367-468, murine)	AA 95-218 (human)	[60,61]
TAX1BP1	Y2H, COIP (overexpression)	C-terminal ZnF-domain (AA 468-699, murine) (AA 373-790, human)		[60,129]
TRIF	COIP (overexpression)			[54]
TBK1	COIP (overexpression)	N- and C-terminus (AA 1-378 and AA 379-790, human)		[55]
IKKε	COIP (overexpression)	N- and C-terminus (AA 1-378 and AA 379-790, human)		[55]
IKK1	COIP (overexpression)	C-terminal ZnF-domain (AA 387-790, human)		[57]
TRAF1	Y2H, COIP (overexpression, endogenous)	N-terminal domain (AA 1-386, human)	TRAF-N domain	[58]
TRAF2	Y2H, COIP (overexpression)	N-terminal domain (AA 1-386, human)	TRAF-N domain (AA 264-358)	[58]
TRAF6	COIP (overexpression)			[59]
ABIN-1	Y2H, COIP (overexpression)	C-terminal ZnF-domain (AA 369-775, murine)	AA 420-647, containing AHD1	[48,130]
ABIN-2	Y2H, COIP (overexpression)	C-terminal ZnF-domain (AA 369-775, murine)	AA 251-430, containing AHD1	[131]
ABIN-3	COIP (overexpression)	C-terminal ZnF-domain	AHD1	[132]
MALT1	COIP (endogenous)		C-terminal part, containing the caspase-like domain	[71]
Ymer	Y2H, COIP (overexpression, endogenous)	N-terminal domain (AA 91-305) containing the OTU-domain	AA 65-134 containing the ubiquitin-binding region	[133]
RNF11	COIP (overexpression, endogenous upon TNF stimulation)		RING-H2 and PPXY motif	[68]
Caspase 8	COIP (overexpression)			[40]

Recently, A20 was found to use also alternative mechanisms to interfere with NF-κB activation [69]. More specifically, upon LPS or IL-1 stimulation, binding of the E3 ligase TRAF6 to A20 prevents TRAF6 from interacting with the E2 conjugating enzymes Ubc5 and Ubc13, which normally triggers TRAF6 autoubiquitination and activation. Similarly, A20 disrupts the interaction between the E2 enzyme Ubc13 and the E3 ligases cIAP1 and TRAF2 upon TNF stimulation, as well as Ubc5/TRAF6 binding in response to IL-1. Moreover, A20 also interacts with Ubc5 and Ubc13 and triggers their K48-polyubiquitination and proteasomal degradation at later time points. Remarkably, reconstitution of *A20*$^{-/-}$ MEFs with A20 carrying mutations in respectively its OTU domain or ZnF4 indicated that both are somehow involved in controlling E2–E3 enzyme interactions and E2 degradation. Clearly, knockin mice with a mutation in the OTU active site or specific ZnFs will be very useful to reveal the DUB-dependent and -independent functions of A20 under physiological conditions.

4.4. Mechanisms regulating A20 activity

As mentioned before, an important mechanism regulating A20 activity is the regulation of its expression. Most cells express no detectable or only limited amounts of A20 under basal conditions and A20 expression is rapidly induced upon NF-κB activation. However, T-cells and B-cells constitutively express considerable high levels of A20, which inhibits ubiquitination of the NF-κB signaling protein MALT1 [70]. In this case, antigen receptor stimulation triggers a rapid decrease and subsequent reappearance of A20, suggesting that A20 removal could be a crucial step in eliminating the negative regulatory activity of A20. Proteasomal degradation of A20, as well as its cleavage by the paracaspase MALT1, contribute to the decrease in A20, resulting in enhanced IL-2 production and NF-κB activation [70,71].

A20 activity can also be regulated by phosphorylation. TNF as well as LPS stimulation leads to the phosphorylation of human A20 at Ser381 by IKK2, increasing the ability of A20 to inhibit NF-κB signaling [72]. A20 phosphorylation might thus allow the down-regulation of NF-κB activity to be tailored more precisely to the needs of the cell, depending on the strength and duration of the inflammatory signal. The mechanism by which phosphorylation modulates A20 function is not known.

The activity of A20 is also regulated by its binding to specific proteins that have essential functions in limiting cytokine- and LPS-induced NF-κB activation and inflammation. The A20-binding protein TAX1BP1 interacts with RIP1 and TRAF6 and recognizes K63-polyubiquitin chains through a C-terminal ZnF domain. Therefore, TAX1BP1 most likely functions as an 'ubiquitin receptor' protein that connects A20 with ubiquitinated substrates [73,74]. Similarly, A20 also requires TAX1BP1 to terminate IRF3 signaling, where TAX1BP1 has been proposed to function as an adaptor molecule between A20 and TBK1/IKKε, preventing the interaction of the E3 ubiquitin ligase TRAF3 with its cognate substrates [75]. Consistent with its role in mediating A20 function, *TAX1BP1*$^{-/-}$ mice die from inflammatory cardiac valvulitis and are hypersensitive to the toxic effects of low doses of TNF and IL-1 [73]. In contrast, Shembade et al. reported that *TAX1BP1*$^{-/-}$ mice are embryonic lethal [74]. It should be mentioned that in the latter study, *TAX1BP1*$^{-/-}$ mice were generated from a gene trap clone, which might explain

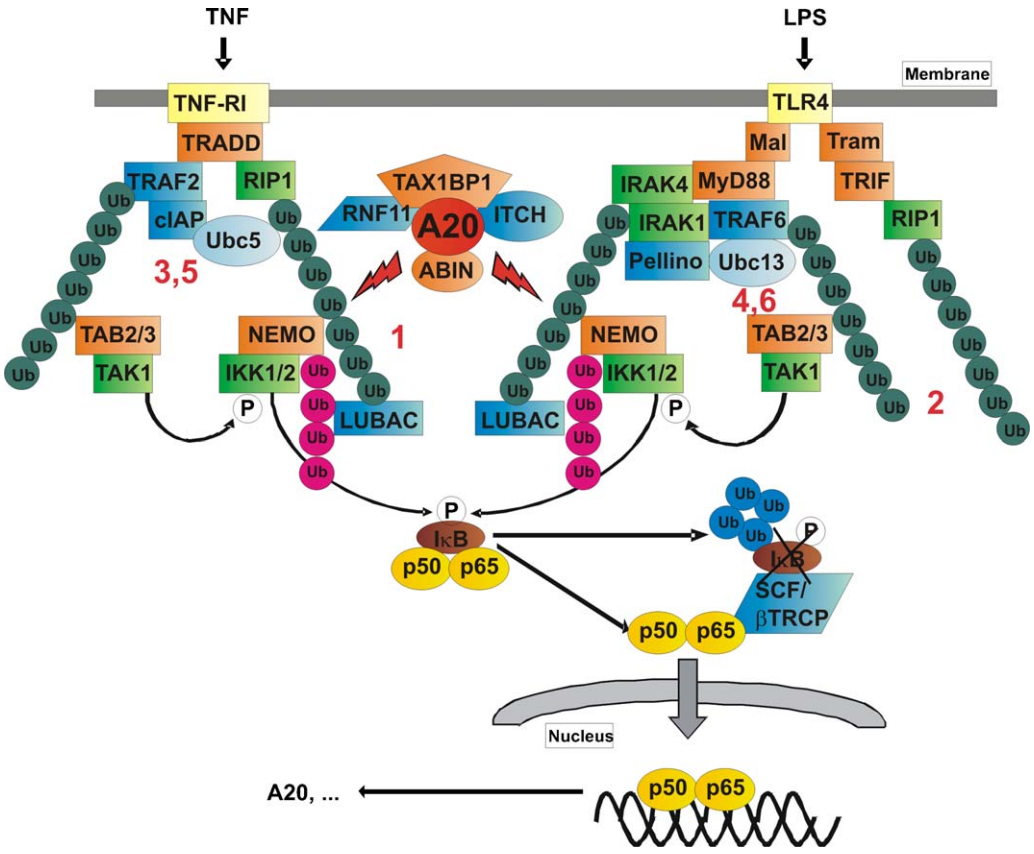

Fig. 4. Inhibition of TNF and LPS-induced NF-κB signaling by A20. NF-κB signaling involves the interaction of multiple proteins, which can be subdivided in adaptors (orange), kinases (green), and E3 ubiquitin ligases (blue). The latter can mediate three different types of polyubiquitination: K63 linked, K48 linked, linear (depicted in different colors). Binding of TNF or LPS to respectively TNF-RI or TLR4 induces the recruitment of different adaptor proteins to the receptor. These form a platform for the binding of E3 ubiquitin ligases and kinases. The E3 ubiquitin ligases together with E2 ligases (Ubc5 and Ubc13) then mediate the K63 polyubiquitination of themselves and the kinases RIP1 and IRAK1. This forms a signal for the recruitment of specific ubiquitin binding adaptor proteins that function to recruit the kinases TAK1 and IKK, resulting in TAK-1 mediated IKK phosphorylation and activation. Similarly, the ubiquitin ligase complex LUBAC is recruited and is responsible for the modification of the adaptor protein NEMO with a linear polyubiquitin chain of which the function is still unclear. Activated IKK then phosphorylates the NF-κB inhibitor protein IκBα, leading to its K48-polyubiquitination by the SCF/βTRCP ubiquitin ligase complex. This type of modification is recognized by the proteasome and results in IκBα degradation, thus allowing NF-κB (depicted here as a p50/p65 dimer) to translocate to the nucleus. One of the many NF-κB responsive genes encodes the NF-κB inhibitor protein A20, which provides a negative feedback mechanism. A20 is a deubiquitinase that inhibits NF-κB signaling by multiple mechanisms: (1) ubiquitin-editing of RIP1 (K63-deubiquitination followed by its K48-polyubiquitination, resulting in RIP1 degradation); (2) K63-deubiquitination of TRAF6; (3,4) disruption of the interaction of the E3 ubiquitin ligases cIAP and TRAF6 with the E2 enzymes Ubc5 and Ubc13; (5,6) K48-polyubiquitination of the E2 enzymes Ubc5 and Ubc13, leading to their proteasomal degradation. A20 binds to specific ubiquitin binding adaptor proteins (ABINs, TAX1BP1), which recruit A20 to specific ubiquitinated substrates or mediate the binding of other proteins such as the E3 ubiquitin ligases RNF11 and ITCH, which are essential for A20 functioning.

the different phenotype. TAX1BP1 and A20 also form a complex with the E3 ubiquitin ligases Itch and RNF11, which are required for A20 to inactivate NF-κB signaling [67,68]. Interestingly, the human T cell leukemia virus type I (HTLV-I) Tax oncoprotein disrupts formation of the A20-TAX1BP1-Itch ternary complex, which may allow constitutive NF-κB activation in HTLV-I infected cells [67]. Finally, A20 also interacts with ABIN-1, -2, and -3. Because overexpression of ABINs mimics the NF-κB inhibitory function of A20, they have been proposed to cooperate with A20 in the regulation of NF-κB signaling [76]. ABINs also function as ubiquitin-binding proteins via a conserved UBAN domain and ABIN-1 has been shown to interact with polyubiquitinated NEMO [77]. Since small interfering RNA targeting of ABIN-1 abrogates A20-dependent deubiquitination of NEMO and RNA interference of A20 impairs the ability of ABIN-1 to inhibit NF-κB activation [65], ABIN-1 has been suggested to physically link A20 to NEMO, hereby facilitating A20-mediated deubiquitination of NEMO, resulting in inhibition of NF-κB. However, ABIN-1 or ABIN-2 knockout mice did not confirm a role for ABIN-1 and -2 in the regulation of NF-κB signaling [78,79], possibly due to redundancy of different ABINs. In contrast, ABIN-1 knockout mice confirmed the previously described anti-apoptotic function of ABIN-1 [78,80]. Although the anti-apoptotic effect of ABIN-1 makes it tempting to speculate that ABIN-1 mediates the anti-apoptotic effect of A20, this was also ruled out [78].

4.5. Other functions of A20 (see Table 2)

Experiments testing the effects of A20 overexpression or deficiency suggest that A20 has functions in addition to the negative regulation of pro-inflammatory gene expression. For example, *A20*$^{-/-}$ mice show thickening of epidermal and dermal layers without apparent inflammation, suggesting a role for A20 in skin differentiation [10]. Consistent with this idea, A20 is found in the basal layer of the forming skin at 15 dpc, and in epithelial cells at epidermal invaginations at dpc 17, suggesting a role for A20 in early hair follicle development [5]. As *IκBα*$^{-/-}$ mice have a similar skin phenotype [81] to *A20*$^{-/-}$ mice, A20 may affect skin differentiation via NF-κB regulation. However, IKK1, which interacts with A20 [57], controls skin development independently of NF-κB activation, by stimulating keratinocyte differentiation [82,83]. Therefore, it is possible that A20 affects this NF-κB-independent function of IKK1 to modulate skin differentiation.

A20 expression has also been shown to exert anti-proliferative effects on cells. Adenoviral transfection of A20 inhibits vascular

smooth muscle cell (VSMC) proliferation induced by TNF by arresting cells in the G_0/G_1 phase [84]. In VSMCs transfected with A20, expression of p53 and the p53 target genes $p21^{waf1}$ and $p27^{kip}$ is increased [85], which would be expected to contribute to the anti-proliferative effect of A20. The effect of A20-deficiency on cell proliferation has not yet been tested, which is essential to confirm that A20 regulates cell proliferation under physiological conditions. Recent data suggest that A20 is involved in neovascularisation. A20 knockdown in HUVEC cells reduces both tubule area and length in *in vitro* angiogenesis assays [86]. Furthermore, A20 expression is largely restricted to endothelial cells of new vasculature in breast cancers [86]. It is unclear whether either of these novel functions of A20 is due to elevated NF-κB activity.

5. Conclusions and future perspectives

Immune signaling pathways are highly complex and tight control is needed in order to maintain homeostasis. The NF-κB pathway serves as a paradigm and has drastically increased our understanding of signal propagation and termination mechanisms. Part of the complexity comes from the many posttranslational modifications which occur. Of these, polyubiquitination has emerged as an exciting area of regulation for NF-κB signaling, and it can be expected that other signaling pathways use similar regulatory mechanisms. Mounting evidence indicates that A20 interferes with NF-κB, IRF3 and cell death signaling to limit inflammation and immunity by modulating the ubiquitination status of specific signaling proteins via multiple mechanisms. What determines whether A20 removes K48- or K63-polyubiquitin chains, as well as the underlying mechanism of its ubiquitin ligase activity remain intriguing questions. Posttranslational modifications of A20, including phosphorylation and proteolytic processing, appear to be critical for its function and activity. It will be of interest to analyze whether A20 can also undergo other posttranslational modifications and if there is cross-talk between the different A20 modifications.

A20 has been associated with several diseases. Importantly, polymorphisms in the A20 genomic region (in most cases located outside the A20 protein encoding sequence) are associated with autoimmunity [3]. Moreover, A20 is inactivated in a substantial number of lymphomas by deletion, promoter methylation, frame shift mutations and/or non-sense mutations that result in truncations and point mutations in A20 [2,87–89]. Because both A20 alleles are often affected in these lymphomas, A20 was proposed as a new tumor suppressor. It will be important to study the effect of the corresponding mutations on the expression and biological activity of A20, using several cellular and biochemical assays. As many mutations in the A20 locus are located outside the mRNA encoding region, also epigenetic regulation of A20 expression is likely to be an important issue. Finally, what is the relationship of A20 with other NF-κB regulating DUBs such as CYLD, which shares several substrates with A20? This question, as well as improved understanding of the physiological functions of A20, might be addressed by studying the cell type and tissue specific functions of A20, using a combination of mouse genetics, primary cells, cell lines, and patient samples. A detailed understanding of the molecular and cellular function of A20 is likely to facilitate the development of novel therapeutics for inflammatory diseases and cancer.

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