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3 **Expression, biological activities and mechanisms of action of A20**  
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6 **(TNFAIP3)**  
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## Abstract

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3 A20 (also known as TNFAIP3) is a cytoplasmic protein that plays a key role in the negative  
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5 regulation of inflammation and immunity. Polymorphisms in the *A20* gene locus have been  
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7 identified as risk alleles for multiple human autoimmune diseases, and A20 has also been  
8  
9 proposed to function as a tumor suppressor in several human B-cell lymphomas. A20  
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11 expression is strongly induced by multiple stimuli, including the proinflammatory cytokines  
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13 TNF and IL-1, and microbial products that trigger pathogen recognition receptors, such as  
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15 Toll-like receptors. A20 functions in a negative feedback loop, which mediates its inhibitory  
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17 functions by downregulating key proinflammatory signaling pathways, including those  
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19 controlling NF- $\kappa$ B- and IRF3-dependent gene expression. Activation of these transcription  
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21 factors is controlled by both K48- and K63- polyubiquitination of upstream signaling  
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23 proteins, respectively triggering proteasome-mediated degradation or interaction with other  
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25 signaling proteins. A20 turns off NF- $\kappa$ B and IRF3 activation by modulating both types of  
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27 ubiquitination. Induction of K48-polyubiquitination by A20 involves its C-terminal zinc-  
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29 finger ubiquitin-binding domain, which may promote interaction with E3 ligases, such as Itch  
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31 and RNF11 that are involved in mediating A20 inhibitory functions. A20 is thought to  
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33 promote de-ubiquitination of K63-polyubiquitin chains either directly, due to its N-terminal  
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35 deubiquitinase domain, or by disrupting the interaction between E3 and E2 enzymes that  
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37 catalyze K63-polyubiquitination. A20 is subject to different mechanisms of regulation,  
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39 including phosphorylation, proteolytic processing, and association with ubiquitin binding  
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41 proteins. Here we review the expression and biological activities of A20, as well as the  
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43 underlying molecular mechanisms.  
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Keywords: A20; NF- $\kappa$ B; inflammation; autoimmunity; ubiquitination

## 1. Introduction

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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 triggered, leading to the activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B), 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 (Figure 1). Furthermore, A20 also functions as a tumor suppressor in several B cell lymphomas. The role of A20 as a disease associated gene

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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<sup>+</sup>CD8<sup>+</sup> 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.

1 On the other hand, a large number of B-cell lymphomas contain mutations in *A20* that result  
2 in loss of *A20* protein expression, indicative for a tumor suppressor role for *A20* [9], and  
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4 suggesting that the role of *A20* in tumorigenesis might be cell type dependent.  
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7 In mice, constitutive *A20* mRNA expression can be detected in epithelial cells  
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9 involved in early hair follicle development, but the highest levels of *A20* mRNA are seen in  
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11 lymphoid tissues, like thymus, gut-associated lymphoid aggregates (containing B- and T-  
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13 cells) and spleen [5]. Moreover, TNF injection in mice results in a substantial upregulation of  
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15 *A20* mRNA in liver, kidney, spleen, thymus, lung, colon and lymph nodes [10].  
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19 The human *A20* gene is located on chromosome 6q23 [11], while the murine *A20* gene  
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21 is mapped to chromosome 10 at 13 cM [5, 12]. Human *A20* mRNA is 4 kb long, containing  
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23 an open reading frame of 2370 nucleotides, encoding a protein of 790 amino acids with a  
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25 molecular weight of 90 kDa [13]. The 3'-untranslated region contains four copies of the  
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27 sequence ATTTA, an mRNA destabilizing motif that is frequently found in the 3'UTR of  
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29 early response inflammatory mediators. *A20* mRNA is superinduced by cycloheximide [4],  
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31 suggesting a role for protein synthesis in the destabilization of *A20* mRNA. Reporter assays  
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33 indicate that transcriptional activation of the *A20* gene is mediated by two NF- $\kappa$ B binding  
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35 sites in its promoter [14]. Consistently, *A20* expression is reduced in cells genetically  
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37 deficient in NF- $\kappa$ B activation [15]. Furthermore, *A20* negatively regulates its own expression  
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39 as a consequence of its NF- $\kappa$ B inhibitory activity [14], resulting in *A20* mRNA only being  
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41 transiently induced following TNF stimulation [4]. The rapid induction of the *A20* gene upon  
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43 TNF stimulation has been suggested to involve the constitutive association of the general  
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45 transcription apparatus and co-activators, such as CBP and p300, with the *A20* promoter,  
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47 mediated by the transcription factor Sp-1 [16]. While Sp-1 activity is necessary for the  
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49 efficient transcription initiation, NF- $\kappa$ B binding to the promoter enhances the re-initiation  
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51 rate, resulting in multiple transcription rounds and augmenting expression levels. The basal  
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1 activity of the *A20* gene is repressed at the elongation step by DRB sensitivity-inducing factor  
2 (DSIF), whose activity is controlled by a negative upstream promoter element called ELIE  
3 (elongation-inhibitory element) [17]. Remarkably, following stimulation, inhibition of the  
4 *A20* promoter by DSIF persists, but it is now regulated by NF- $\kappa$ B rather than ELIE. Recently,  
5 an E-box, overlapping with ELIE, in the *A20* promoter was demonstrated to bind upstream  
6 stimulatory factor (USF)1 [18]. USF1 mediates DSIF inhibition and recruitment to the *A20*  
7 promoter under basal conditions. Once NF- $\kappa$ B is induced, the E-box is no longer involved in  
8 DSIF inhibition. Consistent with that, USF1 is displaced from the promoter by NF- $\kappa$ B.  
9 (Figure 2)

### 3. *A20* structure

10 The amino acid sequence of *A20* is highly conserved (more than 90% identical when  
11 compared to human *A20*) in various mammalian species, and clear orthologs are present in  
12 Zebrafish and *Xenopus* (50% identical to human *A20*, but up to 90% in the N-terminal *A20*  
13 domain). In contrast, no such clear orthologs can be found in *Drosophila* and *C. elegans* [19].  
14 Homology comparisons have revealed the presence of an ovarian tumor (OTU) domain in the  
15 N-terminal part of *A20* (Figure 3) [20], suggesting that *A20* functions as a deubiquitinase,  
16 which has been confirmed biochemically [21]. The crystal structure of the OTU-domain  
17 shows ten  $\beta$ -strands and ten  $\alpha$ -helices, which are arranged to form an expansive surface on  
18 which ubiquitin binding, interaction with substrates and catalysis can take place [22, 23]. The  
19 catalytic site is composed of a catalytic triad (Cys103, His256, Asp70), with a highly  
20 conserved surface patch, forming the ubiquitin-binding site and a separate region for binding  
21 target proteins. Cezanne and TRABID are two other OTU-domain containing deubiquitinases



1 that show considerable similarity with A20 within their OTU-domain, and have therefore  
2 been proposed as A20-like proteins [24, 25].  
3

4 The C-terminal domain of A20 contains seven zinc finger (ZnF) structures, of which 6  
5 have the sequence  $CX_4CX_{11}CX_2C$  and one is similar to the sequence  $CX_2CX_{11}CX_2C$  (Figure  
6 3) [13]. The human and mouse A20 ZnFs are completely identical, except for a single  
7 substitution of methionine for valine in ZnF4 [5]. It has been proposed that ZnF4 confers  
8 intrinsic ubiquitin ligase activity to A20 (see 4.3). The C-terminal ZnF containing domain is  
9 also required for homo-oligomerization of A20, although it is not clear whether this is  
10 important for A20 function [13]. The A20-related protein Cezanne only contains a single  
11 A20-like zinc finger in its C-terminus, which is most similar to ZnF4 and ZnF7 of A20 [25].  
12 Also the A20-related protein TRABID contains three ZnFs at its N-terminus, but these are  
13 unrelated to those of A20. Finally, A20-like ZnFs have also been described in other proteins  
14 such as ZNF216 and Rabex-5 [26, 27].  
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31 Between ZnF3 and ZnF4 of A20, there is a perfect 14-3-3 binding sequence  
32 (RSKSDP), which is conserved between human and mouse (Figure 3) [28, 29]. Consistently,  
33 A20 specifically interacts with the  $\eta$  and  $\zeta$  isoforms of 14-3-3, as mutations in the consensus  
34 14-3-3 binding site abolish these interactions [28]. Interaction of A20 with 14-3-3 alters the  
35 localization of A20 from a punctuate cytoplasmic staining to a more diffuse cytoplasmic  
36 pattern, which is also associated with decreased amounts of A20 in the insoluble cell fraction  
37 [29]. Mutations in the 14-3-3 binding domain of A20 lead to proteolysis of A20 in MCF-7  
38 cells [30], suggesting that the binding to 14-3-3 protects A20 from degradation. Although  
39 A20 is mostly localized in the cytosol, a fraction of A20 can associate with a lysosome-  
40 interacting compartment in a manner that requires its carboxy terminal ZnFs [31]. This  
41 lysosome-associated A20 has been shown to target the A20-associated signaling molecule  
42 TRAF2 to the lysosomes for degradation [32].  
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## 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 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-

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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, 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

1 fragmentation (RIP1 and caspase 8 dependent and typical for apoptosis). The mechanism for  
2 the anti-necrotic action of A20 is still unknown.  
3

4 In contrast to its cytoprotective effects, A20 has also been reported to sensitize  
5 vascular smooth muscle cells to cytokine- and Fas-mediated apoptosis [35]. In addition, cells  
6 expressing high levels of A20 were shown to be more sensitive to oxidative stress-mediated  
7 cell death than cells with low A20 levels [43]. The reason for these paradoxical effects is still  
8 unclear, but might reflect the delicate balance between a direct anti-apoptotic effect of A20  
9 and an indirect apoptosis-sensitizing effect due to its NF- $\kappa$ B inhibitory function.  
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#### 22 *4.2. Negative regulation of inflammation by A20*

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27 Early experiments in cell lines suggested an anti-inflammatory role for A20, since  
28 overexpressed A20 was found to substantially inhibit NF- $\kappa$ B activation in response to several  
29 different stimuli (see Table 2). Consistently, inhibition of NF- $\kappa$ B activation by A20  
30 expression decreases expression of several NF- $\kappa$ B target genes, including E-selectin, ICAM-  
31 1, IL-8, I $\kappa$ B $\alpha$ , tissue factor, VCAM-1, IL-6 and GM-CSF [44-50]. A physiological function  
32 of A20 in the negative regulation of inflammation was confirmed by the phenotype of A20  
33 knockout mice [10]. A20<sup>-/-</sup> mice die shortly after birth by severe inflammation and tissue  
34 damage in multiple organs, including liver, kidneys, intestine, joints and bone marrow. A20<sup>-/-</sup>  
35 *RAG1*<sup>-/-</sup> mice, which are devoid of T- and B-cells, display the same phenotype, indicating that  
36 A20 regulates innate immune homeostasis independent of adaptive immune cells [51]. In  
37 accordance with A20 overexpression experiments, analysis of MEFs from A20<sup>-/-</sup> mice  
38 demonstrates a role for A20 in regulating NF- $\kappa$ B activation [10]. TNF stimulation of A20-  
39 deficient MEFs results in prolonged IKK activation compared to WT cells. This causes  
40 sustained phosphorylation and degradation of I $\kappa$ B $\alpha$ , and consequent increased NF- $\kappa$ B activity  
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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- $\kappa$ B, independently of TNF. Indeed, recent experiments have suggested that the  
inflammatory phenotype of *A20*<sup>-/-</sup> mice is due to excessive signaling from MyD88-dependent  
TLRs (such as TLR3, TLR4 and TLR9); unlike, *A20*<sup>-/-</sup> mice, *A20*<sup>-/-</sup>*MyD88*<sup>-/-</sup> 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*<sup>-/-</sup> 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].

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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- $\beta$  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/IKK $\epsilon$  kinases,  
possibly involving direct interaction of A20 with TBK1, IKK $\epsilon$  and TRIF. However, analyses  
of *A20*<sup>-/-</sup> 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*<sup>-/-</sup>

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*MyD88*<sup>-/-</sup> mice have higher levels of IFN- $\beta$  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 I $\kappa$ B $\alpha$  phosphorylation is sustained due to prolonged IKK activation. These results suggest that A20 may restrict TRIF-dependent signaling to IFN- $\beta$  by regulating NF- $\kappa$ B activation and not IRF3. A20 may control common signaling proteins involved in MyD88- and TRIF-dependent signaling to NF- $\kappa$ B, but not implicated in IRF3 activation. Possible candidates are TRAF6 and RIP1 (see 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- $\kappa$ B inhibitory effect of A20

As discussed in 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- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B. For example, overexpression of A20 in HEK293T and L929sA cells inhibits TNF-induced NF- $\kappa$ B dependent luciferase expression without affecting nuclear translocation and

1 DNA-binding of NF- $\kappa$ B, suggesting that A20 specifically interferes with an NF- $\kappa$ B  
2 transactivation signal [48, 57]. It will clearly be important to obtain complementary evidence  
3 for A20 regulation of NF- $\kappa$ B transactivation using A20-deficient cells.  
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7 Co-expression experiments have demonstrated that several signaling intermediates  
8 upstream of the IKK complex can bind to A20 (see Table 3). For example, A20 interacts with  
9 TRAF1, TRAF2 and TRAF6, which was shown to be mediated by its N-terminal domain in  
10 the case of TRAF1 and TRAF2 [58, 59]. A20 has also been shown to interact with IKK1 and  
11 NEMO via its C-terminal ZnF domain [60, 61], but not with IKK2 [57]. In accordance with  
12 these data, endogenous A20 is recruited to the TNF-RI signaling complex after TNF  
13 stimulation [61]. The ability of A20 to bind to proximal components of the TNF-RI signaling  
14 pathway to NF- $\kappa$ B is consistent with data indicating that A20 regulates IKK activation after  
15 TNF stimulation.  
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30 Wertz et al. proposed that A20 contains two ubiquitin-editing domains, an N-terminal  
31 deubiquitinating (DUB) domain of the OTU family and a C-terminal ubiquitin ligase domain  
32 [62]. The N-terminal domain was shown to deubiquitinate K63-polyubiquitinated RIP1, an  
33 essential mediator of the proximal TNF-RI signaling complex. The C-terminal ZnF containing  
34 domain functions as an ubiquitin ligase that catalyzes the addition of K48-polyubiquitin  
35 chains to RIP1, thereby targeting RIP1 for proteasomal degradation [62, 63] (Figure 4). A20  
36 mutated at the OTU-domain catalytic cysteine residue is defective in adding K48-  
37 polyubiquitin chains to RIP1, suggesting that RIP1 K63 deubiquitination is a prerequisite for  
38 A20-mediated RIP1 K48-polyubiquitination and subsequent degradation. The catalytic  
39 cysteine is also necessary for deubiquitination of K63-polyubiquitinated TRAF6 by A20  
40 (Figure 4) and inhibition of NF- $\kappa$ B activation after LPS stimulation [51]. Furthermore,  
41 stimulation of NOD2 with MDP leads to K63-polyubiquitination of RIP2, which can be  
42 inhibited by A20 depending on its OTU domain [64]. A20 has also been reported to remove  
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1 K63-polyubiquitin chains from NEMO in TNF-stimulated cells. However, the role of NEMO  
2 ubiquitination in NF- $\kappa$ B activation is unclear [65, 66]. As discussed earlier, *in vitro* the N-  
3 terminal domain of A20 has been shown to hydrolyse both K48- and K63-polyubiquitin [21],  
4 and hydrolysis of K48-polyubiquitin chains was later shown to be much more efficient than  
5 K63-polyubiquitin chains [22, 23]. This suggests that other factors such as the  
6 polyubiquitinated protein itself or possibly other A20-binding proteins might determine the  
7 DUB specificity of A20 for K63-polyubiquitinated substrates in intact cells.  
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10 Reconstitution of  $A20^{-/-}$  MEF cells with the OTU catalytic cysteine mutant of A20 is  
11 much less effective in down-regulating TNF-induced NF- $\kappa$ B activity than wild type A20 [62],  
12 suggesting that DUB activity is essential for A20's inhibitory function. However, several  
13 studies showed that the OTU domain is not important for the NF- $\kappa$ B or IRF3 inhibitory  
14 activity of overexpressed A20. For example, overexpression of the C-terminal domain or the  
15 OTU catalytic cysteine mutant of A20 still inhibits TNF-, IL-1- and Pam3Cys-induced NF- $\kappa$ B  
16 activation, as well as poly-(I:C)- and NDV-induced IRF3 activation, as potently as the wild  
17 type molecule [21, 30, 48, 55, 58]. The physiological relevance of such overexpression  
18 experiments remain to be demonstrated.  
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39 ZnF4 is essential for retrovirally expressed A20 to trigger RIP1 proteolysis in  $A20^{-/-}$   
40 MEFs and it has been suggested that ZnF4 confers ubiquitin ligase activity to A20 [62].  
41 However, it is unclear whether A20 has intrinsic E3 ligase activity or if this activity is  
42 mediated by an associated protein such as Itch and RNF11, which are two A20-binding E3  
43 ubiquitin ligases that have been reported to be essential for A20's NF- $\kappa$ B inhibitory function  
44 [67, 68]. It is also evident that ZnF4 is not always required for A20 to inhibit NF- $\kappa$ B. For  
45 example, the inhibitory effect of overexpressed A20 on RIG-I- and virus-induced NF- $\kappa$ B and  
46 IRF3 activation was found to involve ZnF7 and not ZnF4 [53]. Likewise, ZnF7 contributes to  
47 the inhibition of TNF-induced NF- $\kappa$ B activation by overexpressed A20 [37], as well as to the  
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1 localization of A20 to lysosome-associated compartments [31]. Furthermore, oligomerization  
2 of ZnF7 as such inhibits NF- $\kappa$ B activation [37]. Klinkenberg et al. demonstrated that there is  
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4 redundancy between the ZnFs of A20 for inhibition of TNF-induced NF- $\kappa$ B activation, with 4  
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6 ZnFs being sufficient for inhibition [60]. However, it should be noted that either ZnF4 or  
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8 ZnF7 were still present in inhibitory A20 mutants.  
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12 Recently, A20 was found to use also alternative mechanisms to interfere with NF- $\kappa$ B  
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14 activation [69]. More specifically, upon LPS or IL-1 stimulation, binding of the E3 ligase  
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16 TRAF6 to A20 prevents TRAF6 from interacting with the E2 conjugating enzyme Ubc13,  
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18 which normally triggers TRAF6 autoubiquitination and activation. Similarly, A20 disrupts the  
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20 interaction between the E2 enzyme Ubc5 and the E3 ligases cIAP1 and TRAF2 upon TNF  
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22 stimulation, as well as Ubc5/TRAF6 binding in response to IL-1. Moreover, A20 also  
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24 interacts with Ubc5 and Ubc13 and triggers their K48-polyubiquitination and proteasomal  
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26 degradation at later time points. Remarkably, reconstitution of  $A20^{-/-}$  MEFs with A20 carrying  
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28 mutations in respectively its OTU domain or ZnF4 indicated that both are somehow involved  
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30 in controlling E2-E3 enzyme interactions and E2 degradation. Clearly, knockin mice with a  
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32 mutation in the OTU active site or specific ZnFs will be very useful to reveal the DUB-  
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34 dependent and -independent functions of A20 under physiological conditions.  
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#### 44 *4.4. Mechanisms regulating A20 activity*

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49 As mentioned before, an important mechanism regulating A20 activity is the  
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51 regulation of its expression. Most cells express no detectable or only limited amounts of A20  
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53 under basal conditions and A20 expression is rapidly induced upon NF- $\kappa$ B activation.  
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55 However, T-cells and B-cells constitutively express considerable high levels of A20, which  
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57 inhibits ubiquitination of the NF- $\kappa$ B signaling protein MALT1 [70]. In this case, antigen  
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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- $\kappa$ B 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- $\kappa$ B signaling [72]. A20 phosphorylation might thus allow the downregulation of NF- $\kappa$ B 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- $\kappa$ B 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/IKK $\epsilon$ , preventing the interaction of the E3 ubiquitin ligase TRAF3 with its cognate substrates [75]. Consistent with its role in mediating A20 function, *TAX1BP1*<sup>-/-</sup> 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*<sup>-/-</sup> mice are embryonic lethal [74]. It should be mentioned that in the latter study, *TAX1BP1*<sup>-/-</sup> mice were generated from a gene trap clone, which might explain the different phenotype. TAX1BP1 and A20 also form a complex with the E3 ubiquitin ligases Itch and RNF11, which are

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

#### 44 *4.5. Other functions of A20 (see table 2)*

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49 Experiments testing the effects of A20 overexpression or deficiency suggest that A20  
50 has functions in addition to the negative regulation of pro-inflammatory gene expression. For  
51 example, *A20*<sup>-/-</sup> mice show thickening of epidermal and dermal layers without apparent  
52 inflammation, suggesting a role for A20 in skin differentiation [10]. Consistent with this idea,  
53 A20 is found in the basal layer of the forming skin at 15 dpc, and in epithelial cells at  
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1 epidermal invaginations at dpc 17, suggesting a role for A20 in early hair follicle  
2 development [5]. As  $I\kappa B\alpha^{-/-}$  mice have a similar skin phenotype [81] to  $A20^{-/-}$  mice, A20 may  
3 affect skin differentiation via NF- $\kappa$ B regulation. However, IKK1, which interacts with A20  
4 [57], controls skin development independently of NF- $\kappa$ B activation, by stimulating  
5 keratinocyte differentiation [82, 83]. Therefore, it is possible that A20 affects this NF- $\kappa$ B-  
6 independent function of IKK1 to modulate skin differentiation.  
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10 A20 expression has also been shown to exert anti-proliferative effects on cells.  
11 Adenoviral transfection of A20 inhibits vascular smooth muscle cell (VSMC) proliferation  
12 induced by TNF by arresting cells in the  $G_0/G_1$  phase [84]. In VSMCs transfected with A20,  
13 expression of p53 and the p53 target genes p21<sup>waf1</sup> and p27<sup>kip</sup> is increased [85], which would  
14 be expected to contribute to the anti-proliferative effect of A20. The effect of A20-deficiency  
15 on cell proliferation has not yet been tested, which is essential to confirm that A20 regulates  
16 cell proliferation under physiological conditions. Recent data suggest that A20 is involved in  
17 neovascularisation. A20 knockdown in HUVEC cells reduces both tubule area and length in  
18 *in vitro* angiogenesis assays [86]. Furthermore, A20 expression is largely restricted to  
19 endothelial cells of new vasculature in breast cancers [86] It is unclear whether either of these  
20 novel functions of A20 is due to elevated NF- $\kappa$ B activity.  
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## 44 **5. Conclusions and future perspectives**

45 Immune signaling pathways are highly complex and tight control is needed in order to  
46 maintain homeostasis. The NF- $\kappa$ B pathway serves as a paradigm and has drastically increased  
47 our understanding of signal propagation and termination mechanisms. Part of the complexity  
48 comes from the many posttranslational modifications which occur. Of these,  
49 polyubiquitination has emerged as an exciting area of regulation for NF- $\kappa$ B signaling, and it  
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can be expected that other signaling pathways use similar regulatory mechanisms. Mounting evidence indicates that A20 interferes with NF- $\kappa$ B, 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- $\kappa$ B 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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## Figure legends

### Figure 1: A20 is a susceptibility gene for multiple diseases

### Figure 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- $\kappa$ B, 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- $\kappa$ B is activated and binds to the  $\kappa$ B sites in the A20 promoter, leading to re-initiation and multiple rounds of transcription. In addition, NF- $\kappa$ B displaces USF1 from the promoter and takes over the control of DSIF activity, permitting mRNA elongation by RNA polymerase II.

### Figure 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.

### Figure 4: Inhibition of TNF and LPS-induced NF- $\kappa$ B signaling by A20

NF- $\kappa$ B 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

1 polyubiquitin chain of which the function is still unclear. Activated IKK then phosphorylates  
2 the NF- $\kappa$ B inhibitor protein I $\kappa$ B $\alpha$ , leading to its K48-polyubiquitination by the SCF/ $\beta$ TRCP  
3 ubiquitin ligase complex. This type of modification is recognized by the proteasome and  
4 results in I $\kappa$ B $\alpha$  degradation, thus allowing NF- $\kappa$ B (depicted here as a p50/p65 dimer) to  
5 translocate to the nucleus. One of the many NF- $\kappa$ B responsive genes encodes the NF- $\kappa$ B  
6 inhibitor protein A20, which provides a negative feedback mechanism. A20 is a  
7 deubiquitinase that inhibits NF- $\kappa$ B signaling by multiple mechanisms: (1) ubiquitin-editing of  
8 RIP1 (K63-deubiquitination followed by its K48-polyubiquitination, resulting in RIP1  
9 degradation); (2) K63-deubiquitination of TRAF6; (3,4) disruption of the interaction of the E3  
10 ubiquitin ligases cIAP and TRAF6 with the E2 enzymes Ubc5 and Ubc13; (5,6) K48-  
11 polyubiquitination of the E2 enzymes Ubc5 and Ubc13, leading to their proteasomal  
12 degradation. A20 binds to specific ubiquitin binding adaptor proteins (ABINs, TAX1BP1),  
13 which recruit A20 to specific ubiquitinated substrates or mediate the binding of other proteins  
14 such as the E3 ubiquitin ligases RNF11 and ITCH, which are essential for A20 functioning.  
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**Table 1: Expression pattern of A20**

<b>Stimulus</b>	<b>Cell type</b>	<b>Ref.</b>
<b>TNF</b>	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-E1 cell line	[93]
	Primary human hepatocytes, hepatocellular carcinoma cell line HepG2	[94]
	MCF-7 breast carcinoma cell line	[33]
	$\beta$ cells (primary islets, Min6)	[95]
<b>IL-1</b>	$\beta$ cells (primary islets, Min6)	[95]
<b>LPS</b>	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]
<b>CpG DNA</b>	Enterocytes (IEC-18)	[99]
<b>Flagellin</b>	Enterocytes (HCT-15, HT-29, mouse primary cells, Colon-26)	[98]
<b>CD40</b>	B-cells	[100]
	Fibroblast cell line SV80	[101]
<b>TPA</b>	Jurkat T-cells, U937 promonocytic cells	[102]
	Freshly isolated human blood monocytes	[103]
<b>TRAIL</b>	MCF-7	[104]
<b>Angiotensin II</b>	Human adrenocortical cells	[105]
<b>Peptidoglycan</b>	Primary human airway epithelial cells	[96]
<b>H<sub>2</sub>O<sub>2</sub></b>	HeLa cells	[43]
<b>Serum from patients with burn injuries</b>	Human umbilical vein endothelial cells (HUVEC)	[50]

<b>TNF-RII</b>	Murine CT6 cells	[58]
<b>Androgen</b>	LNCaP prostate cancer cells	[106]
<b>CD3</b>	Do-11.10 T cell hybridomas, CD4 <sup>+</sup> and CD8 <sup>+</sup> T-cells	[39]
<b>Vitamin D<sub>3</sub></b>	Increase of expression: * rat insulin-producing $\beta$ -cells (RINm5F) * human islet cells * liver allografts Decrease of expression: * LNCaP cells	[107, 108] [106]
<b>Zinc depletion</b>	Diminished LPS- and TPA-induced expression in promyelocytic leukemia HL-60 cells	[109]
<b>ER stress</b>	Mesangial cells, NRK-52E cells	[110]
<b>Epstein-Barr virus latent membrane protein LMP1</b>	B-cells Human epithelial cells	[111] [112]
<b>Human T-cell leukemia virus type I oncoprotein Tax</b>	Jurkat T cells	[102]
<b>Human cytomegalovirus</b>	Human peripheral blood monocytes	[113]
<b>Influenza virus</b>	Human bronchial epithelial cells, lung homogenates from mice	[114]
<b>Sendai virus</b>	U937	[54]
<b>Measles virus P protein</b>	Monocytes (THP1, U937)	[115]
<b>Hepatitis C virus core protein</b>	HepG2	[116]
<b>Helicobacter pylori</b>	Human gastric cancer cells	[117]
<b>Uropathogenic Escherichia coli</b>	Mice bladder tissues	[118]
<b>VLA-4 stimulation</b>	Monocytes	[119]

**Table 2: Biological activities of A20**

Function	Stimulus	Cells	Evidence	Mechanisms	Ref
<b>Anti-apoptotic</b>	TNF	Thymocytes, MCF7S1, WEHI-S, endothelial cells, $\beta$ -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 <sub>2</sub> O <sub>2</sub>	Lymphoblastoid cell lines	RNAi		[124]
	<b>Anti-necrosis</b>	TNF	L929	overexpression	* inhibition of phospholipases; production of reactive oxygen species
Complement		Endothelial cells	overexpression		[35]
Hypoxia-		Mouse hepatocytes (NMuLi)	overexpression	* increase in PPAR $\alpha$ expression	[125]

	reoxygenation				
	H <sub>2</sub> O <sub>2</sub>	Mouse hepatocytes (NMuLi)	overexpression	* increase in PPAR $\alpha$ expression	[125]
<b>Pro-apoptotic</b>	Fas-and cytokine mediated	Vascular smooth muscle cells (VSMC)	overexpression	*NF- $\kappa$ B inhibition	[43, 126]
<b>NF-<math>\kappa</math>B inhibition</b>	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 LPS	MCF7S1, HEK293T, human airway epithelial cells, HEK293, BAEC, macrophages	overexpression, knockout	* deubiquitination of TRAF6 * inhibition of TRAF6 E3 ligase activity by antagonizing the interaction with E2 enzymes * induction of proteasomal degradation of E2 enzymes	[34, 44, 51, 56, 58, 96] [69]
	H <sub>2</sub> O <sub>2</sub>	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]
<b>AP-1 inhibition</b>	TNF	MCF7S1	overexpression		[34]
	IL-1	MCF7S1	overexpression		[34]
	LMP1	HEK293	overexpression		[127]
	TLR-4	HEK293	overexpression		[56]
<b>IRF3 inhibition</b>	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]
	IKK $\epsilon$ /TBK1 VSV	HEK293	overexpression	*Inhibition of IKK $\epsilon$ /TBK1 ubiquitination by disrupting their interaction with TRAF3 E3 ligase	[75]
<b>Skin differentiation</b>			knockout mice	* impaired NF- $\kappa$ B activation * interaction with IKK1	[5, 10, 57]
<b>Cell cycle arrest</b>		VSMC	overexpression	* arrest in G <sub>0</sub> /G <sub>1</sub> by inhibition of PI3K/Akt/GSK3 $\beta$ signaling * upregulation of p53, p21 <sup>waf1</sup>	[84, 85]

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			and p27 <sup>kip</sup>	
<b>Mitogenic in the liver</b>			adenoviral gene delivery in mice	[94]
<b>Neovascularisation</b>	HUVEC		RNAi	[86]

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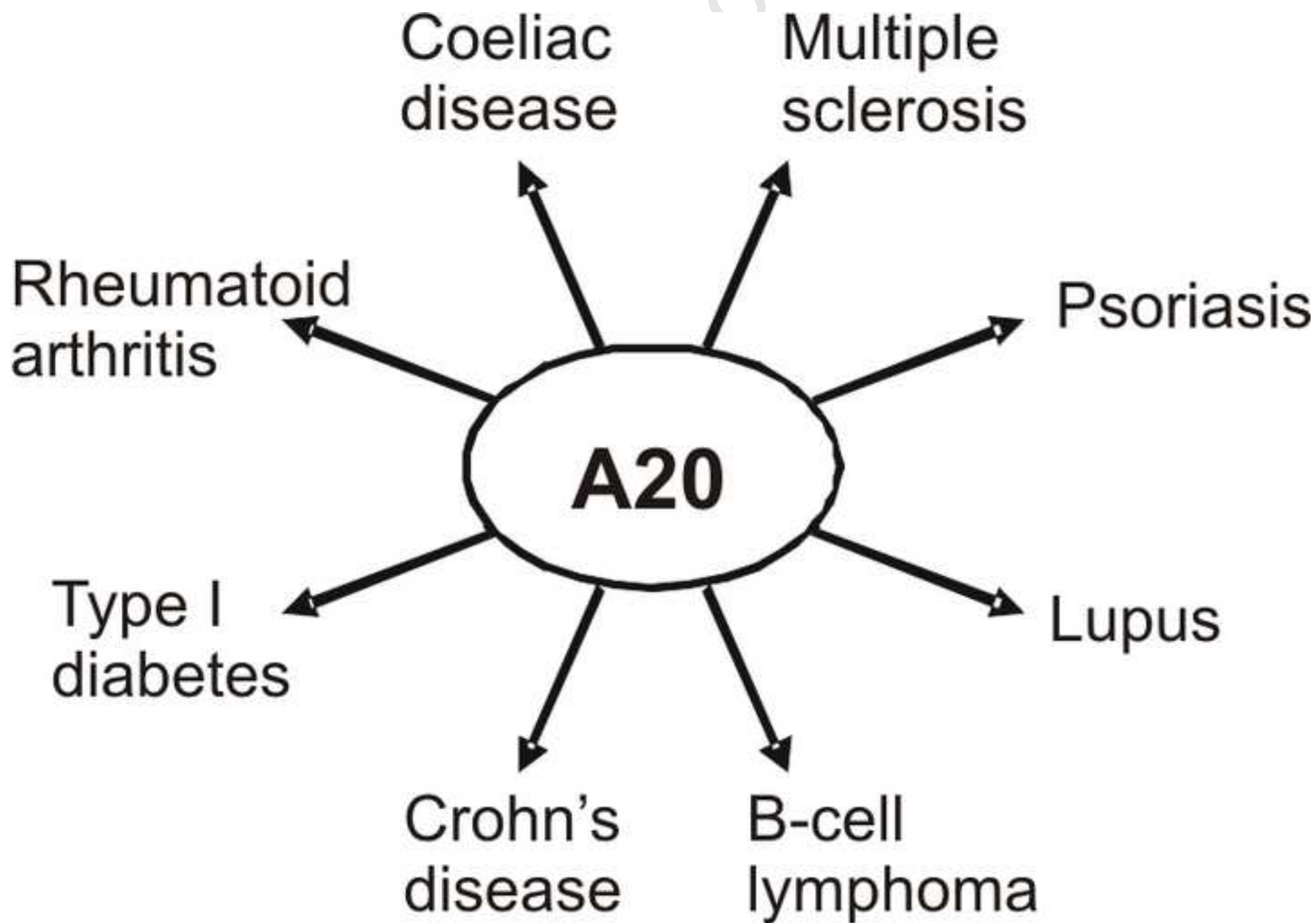
**Table 3: A20-interacting proteins**

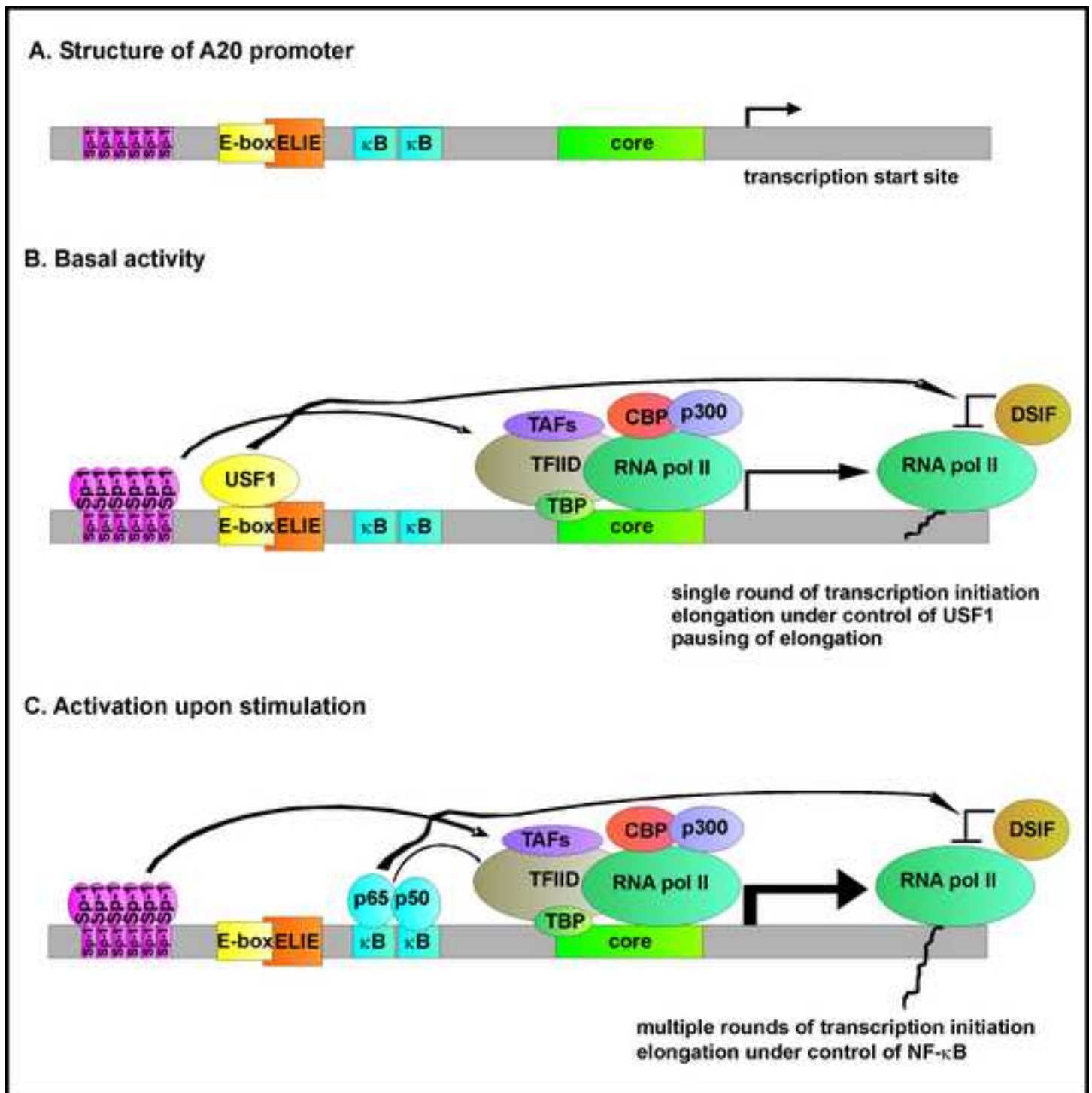
<b>Interacting protein</b>	<b>Evidence</b>	<b>Interacting domain in A20</b>	<b>Interacting domain in partner protein</b>	<b>Ref</b>
A20	Y2H	C-terminal ZnF-domain (AA 373-790, human)		[128]
14-3-3	Y2H, in vitro binding assay, COIP (overexpression, semi-endogenous)	RSKSDP sequence (human and murine) between 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]
IKK $\epsilon$	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]



Figure 1





Lys63 deubiquitination

Lys48 ubiquitination

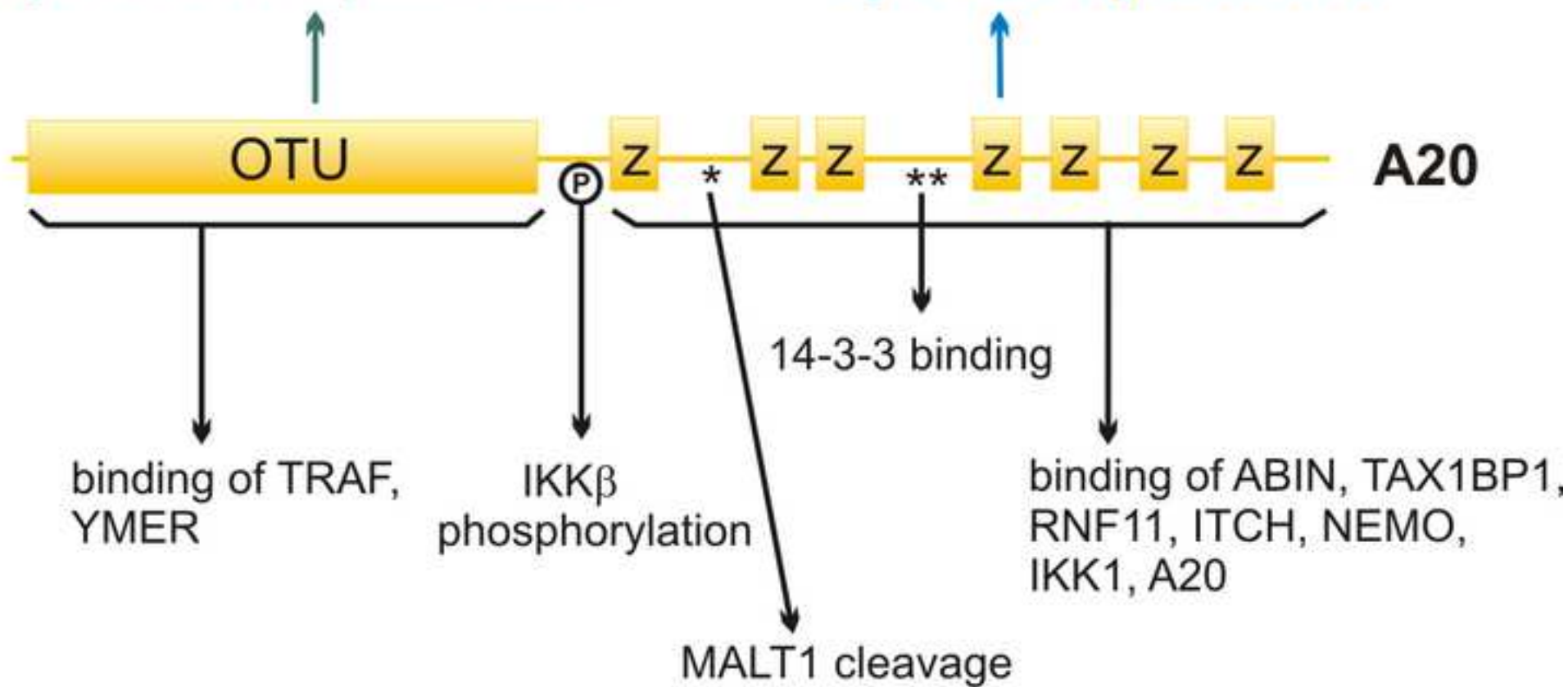


Figure 4

