Significant functional difference between *TNFAIP3* truncation and missense mutants

A20, encoded by *TNFAIP3*, is a molecular "brake" of the canonical NF-κB activation pathway and attenuates the NF-κB activity triggered by a number of surface receptors.[1,2] A20 contains an N-terminal OTU domain that possesses deubiquitinating activity, and 7 zinc finger (ZF) domains in its C-terminus that confers the E3 ubiquitin ligase activity.[3] Through removing the K63-linked ubiquitin chain, catalysing the K48-linked polyubiquitination, and also direct binding to the linear polyubiquitin chain of its targets, A20 can inactivate a number of NF-κB positive regulators, including RIP1/2, TRAF6, Ubc13 and NEMO, thus negatively regulating the signalling of several surface receptors, including BCR, TNFR, TLR and IL1βR.[1,2,4] A20 itself is a transcriptional target of NF-κB, and its transcriptional activation by NF-κB thus serves as an auto-negative feedback to attenuate NF-κB activities triggered by these receptor signallings.[1,2]

In view of its central role in NF-κB regulation, it is unsurprising that A20 abnormalities are linked to a wide spectrum of human diseases. *A20* inactivation by deletion and/or mutation is frequently seen in MALT lymphoma of the ocular adnexa and salivary gland, diffuse large B-cell lymphoma (DLBCL), Hodgkin lymphoma and primary mediastinal B-cell lymphoma.[5-10] In addition, *A20* is linked to a range of chronic inflammatory disorders including autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis,[1,2] which is associated with a significantly increased risk of lymphoma.[11]

The majority of *A20* somatic mutations seen in various lymphoma subtypes are frameshift indels and nonsense changes, with the remaining mutations (~10%) being missense alterations. While the frameshift indels and nonsense changes cause a truncated A20 product and hence inactivate its activity, the biological impact of missense mutations on A20 function remains unknown. In the present study, we have investigated the functional impact of a range of *A20* mutations using *in vitro* reporter assays, particularly focusing on those occurring on the conserved amino acids across different species.

We have reviewed *A20* missense mutations identified in DLBCL and MALT lymphoma from our previous and ongoing investigations.[12,13] After reviewing their somatic status, whether occurring in a known functional domain and on conserved amino acids across different species, a total of 7 missense mutations were selected for functional investigation (Figure 1A). In addition, three representative *A20* truncation mutants, lacking the seventh ZF domain, both the sixth and seventh ZF domain, or all seven ZF domains, together with wild type A20 were used as control (Figure 1A).

The various truncated forms of *A20* were generated from pIRESpuro2-HA-hA20 by PCR and cloned into the pIRESpuro2-HA vector at the EcoRI and NotI sites. The *A20* mutants containing a single point mutation were generated from the wild type using the QuickChange Lightning Site-directed mutagenesis kit (Stratagene, USA). PCR and sequencing were performed to verify the *A20* sequence and reading frame. These constructs were also tested for their expression by transient transfection of HEK293T and Western blot analysis. The effect of A20 and its various mutants on repression of NF-κB activation by various stimuli (TNFα, MYD88 mutant or CARD11 mutant) was investigated in HEK293T cells using a Dual-Luciferase reporter assay (Promega, UK).[12,14] Briefly, HEK293T cells ($4.5x10^5$) were transfected with 2μg of expression vector (A20, MYD88-S219C or CARD11-F130V), 0.8μg of pNF-κB-luc and 0.2μg of pRL-TK using TransIT®-293 reagent (Mirus, UK). The transfected cells were cultured for 24 hours, where indicated stimulated by TNFα (300IU/ml) for 2 hours, and then harvested for luciferase activity measurement. For each experiment, at least three independent transfections and triplicate reporter assays were performed, and data was presented as a mean ± standard deviation. The difference in reporter activities between the wild type and mutant A20 was analysed using an unpaired student's *t*-test with the GraphPad Prism version 5.00 software (GraphPad Software, San Diego, USA).

Since A20 negatively regulates NF-κB activation triggered by several receptor signalling through inactivating various signalling molecules, we separately tested the effect of A20 mutants on NF-κB activation simulated by TNFR, TLR and BCR signalling.

As expected, the wild type A20 was a potent inhibitor of TNFα induced NF-κB activities, and the three A20 truncation mutants showed a substantial impairment in NF-κB repression. Interestingly, the OTU-ZF6 truncation mutant showed the worst impairment under stimulation of TNFα in comparison with those stimulated by the MYD88 and CARD11 mutants (Figure 1). The seventh ZF domain of A20 is essential for binding to the linear ubiquitin chain of both RIP1 and NEMO, which is critical for A20 function in repression of NF-κB activation. The OTU-ZF6 truncation mutant without the seventh ZF domain may potentially have a more extended effect on NF-κB activation by TNFα than the MYD88 and CARD11 mutant since it most likely loses its ability to inactivate both RIP1 and NEMO in the TNFR signalling, but only at NEMO in the TLR (MYD88 mutant) and BCR (CARD11 mutant) signalling.[15]

To our surprise, none of the seven A20 missense mutants showed any significant loss of NF-κB repression in comparison with the wild type. It is worth noting that the level of expression of the A20 missense mutants was much lower than the A20 truncation mutants, as shown by Western blot (Figure 1B), and the A20 D117V mutant showed the lowest expression level; nonetheless, they were still highly potent in NF-κB repression.

Similarly, the wild type A20 was highly robust in suppression of NF-κB activation by the MYD88-S219C mutant, and all the three A20 truncation mutants showed a significantly impaired capacity in NF-κB repression, though to varying extents; the A20-OTU mutant displayed the most damage (Figure 1C). Among the seven A20 missense mutants, three (D117V, I194T, and C483R) were significantly different from the wild type. Their level of impairment in NF-κB repression, however, was rather modest with the exception of the A20 D117V mutant, which may be due to its low expression level.

The reporter assay using the CARD11-F130V mutant to simulate the BCR signalling showed nearly identical impact of various A20 mutants on NF-κB repression to the above assay using the MYD88-S219C mutant (Figure 1D). Interestingly, Western blot with anti-HA antibody (HA as an N-terminal tag) showed an additional band of 50 kDa in all but the A20-OTU truncation mutant (Figure 1D). Previous studies have clearly established that A20 is cleaved after R439 by MALT1 in response to upstream signalling, generating an N-terminal 50 kDa and a C-terminal 37 kDa fragment.[16] Thus, it is most likely that the CARD11-F130V mutant recruited and activated BCL10 and MALT1, consequently triggering A20 cleavage. As expected, this N-terminal 50 kDa A20 fragment was not

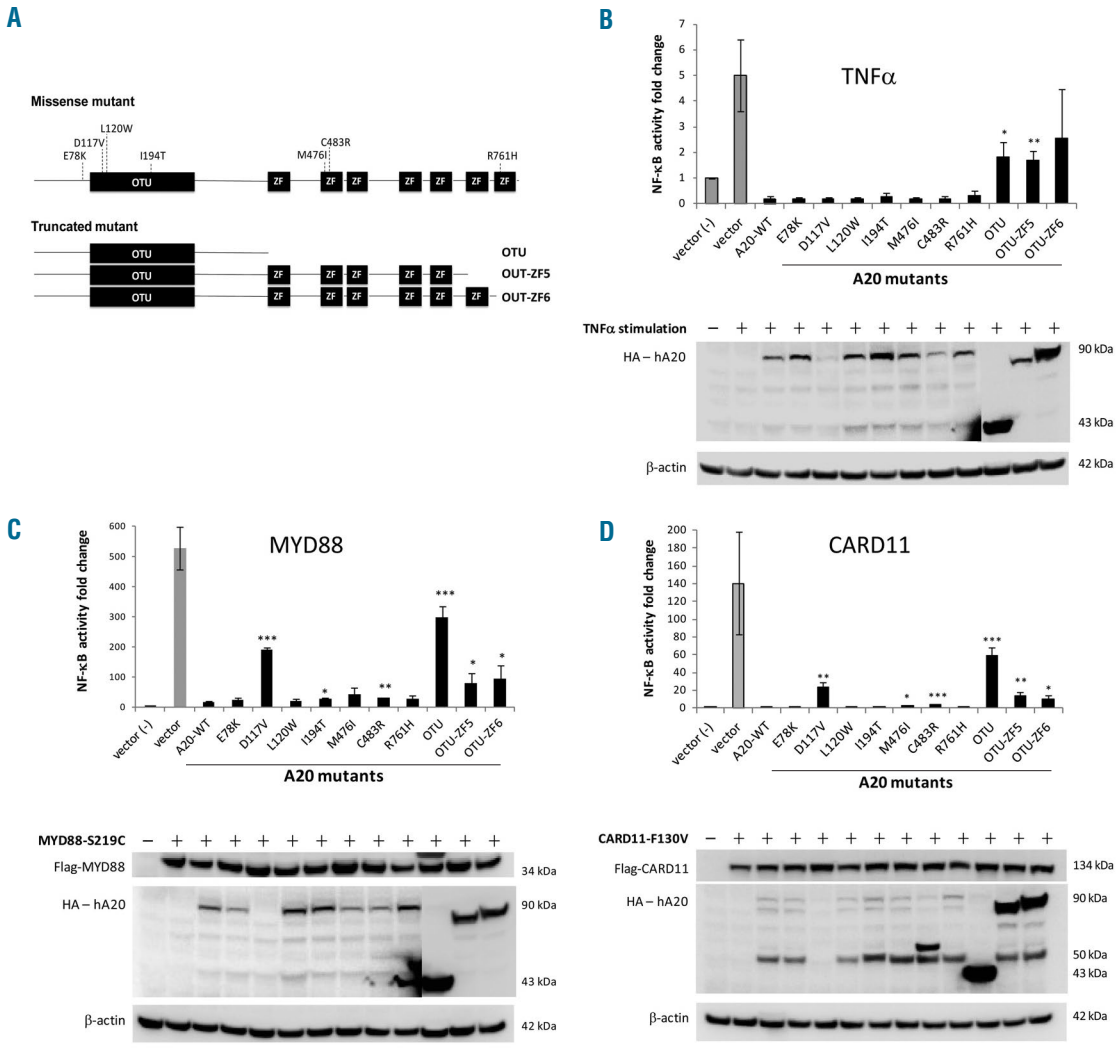

Figure 1. Functional characterisation of *TNFAIP3 (A20)* mutations. (A) The representative A20 mutants investigated by *in vitro* reporter assays, which include three truncation and 7 missense mutations that affect conserved amino acid residues. (B-D) NF-κB reporter assay shows that the wild type A20 is a potent inhibitor of NF-κB activation by TNFα, MYD88 and CARD11 mutants in HEK293T cells. All the three A20 truncation mutants consistently show a substantial impairment in suppression of NF-κB activation triggered by each of the three signalling pathways investigated, while the A20 missense mutants largely retain their ability to act as a global negative regulator of NF-κB despite some of the missense mutants displaying inconsistent evidence of impairment. The data is from three independent experiments and presented as a mean ± standard deviation, and the difference between A20 and its mutants is analysed by the unpaired student's *t*-test. **P*<0.05, ***P*<0.01, ****P*<0.001. There is a significant difference in the level of protein expression between the A20 truncation and missense mutants in the reporter assays under stimulation by the MYD88 mutant and TNFα, and it is not possible to clearly illustrate their expression using the image from the same exposure time. Thus, the image for the A20 truncation mutants was taken from a shorter exposure (5 seconds), while the image for wild type A20 and its missense mutants was from a longer exposure (30 seconds).

detected by Western blot with an antibody against C-terminal A20. Nonetheless, the additional Western analysis further confirmed a weak expression of the full length A20, indicating incomplete cleavage (*data not shown*). Despite such low levels of expression, various A20 missense mutants, with the exception of D117V, were still robust in NF-κB suppression.

In summary, the A20 truncation mutants consistently showed a substantial impairment in suppression of NF-κB activation triggered by each of the three signalling pathways investigated, while the A20 missense mutants largely retained their role of global negative regulator of NF-κB, despite the fact that some of the missense mutants displayed inconsistent evidence of impairment based on the reporter assays. There is, however, a clear functional difference between A20 truncation and missense mutants. It is therefore important to distinguish between these mutations when correlating A20 mutations with biological readout and clinical outcome data.

Leire Escudero-Ibarz, Ming Wang, and Ming-Qing Du

[1]Division of Molecular Histopathology, Department of Pathology, University of Cambridge, UK

Correspondence: mqd20@cam.ac.uk;
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