BCA2 Inhibits the Nuclear Translocation of NF-κB, Impairing HIV-1 Transcription

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ABSTRACT

BCA2/Rabring7 is a Tetherin/BST2 co-factor that promotes the lysosomal degradation of trapped HIV-1 virions, but also functions as a BST2-independent anti-HIV factor by targeting Gag for lysosomal degradation (Fig. 1). Since many antiviral factors regulate the NF-κB innate signaling pathway, we investigated whether BCA2 is also connected to this pro-inflammatory cascade. Here we show that *BCA2* is induced by NF-κB-activating cytokines, and that up-regulation of BCA2 provides a regulatory negative feedback on NF-κB. Specifically, BCA2 serves as an E3 SUMO-ligase in the SUMOylation of IκBα, which enhances the sequestration of NF-κB components in the cytoplasm. Remarkably, the BCA2-mediated inhibition of NF-κB has detrimental effects on HIV-1 transcription and replication. Therefore, our findings indicate that BCA2 represents an important barrier to HIV-1 by affecting multiple steps in its replication cycle: not only does BCA2 prevent assembly and release of nascent virions, but also significantly restricts HIV-1 transcription by inhibiting the NF-κB pathway.

Current model of BCA2-mediated restriction

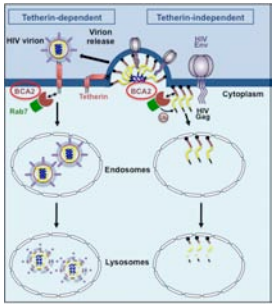


Figure 1. BCA2 has BST2-dependent and independent anti-HIV activity

- BCA2 was identified as a BST2 co-factor in the restriction of HIV-1 by inducing the lysosomal degradation of tethered virions (Miyakawa et al., 2009).
- We later demonstrated that BCA2 also has BST2-independent anti-HIV-1 activity. In particular, BCA2 promotes the ubiquitination and lysosomal degradation of retroviral Gag proteins, impairing virus assembly (Nityanandam and Serra-Moreno 2014).

RESULTS

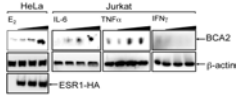


Figure 2. BCA2 is induced by NF-κB-activating cytokines

CD4+T cells were treated with NF-κB-inducing cytokines (IL-6 and TNFα) and non-NF-κB pro-inflammatory cytokines (IFNγ). The expression of endogenous BCA2 was monitored by western blotting. BCA2 up-regulation was only observed in the presence of NF-κB-activating cytokines.

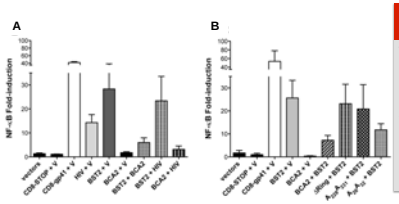


Figure 3. The BCA2-meditated inhibition of the NF-κB pathway requires the catalytic activity of BCA2

To study BCA2's effect on the NF-κB pathway, luciferase assays were performed. HIV-1 proviral DNA, the cytoplasmic domain of gp41 (CD8-gp41) and BST2 were used as positive controls. Overexpression of HA-BCA2 resulted in NF-κB activity levels similar to those observed with the negative controls (Fig. 3A). However, a significant reduction (~ 5-fold) in NF-κB activity was observed in cells co-expressing NF-κB inducers and HA-BCA2 (Fig. 3A). Notably, NF-κB activity was rescued in the presence of BCA2 RING-finger mutants (delta-RING and $A_{228}A_{23}$), and partially restored in the presence of a Zinc-finger mutant ($A_{26}A_{32}$) (Fig. 3B). Thus, **BCA2 requires a functional RING-finger domain to turn off the NF-κB pathway**.

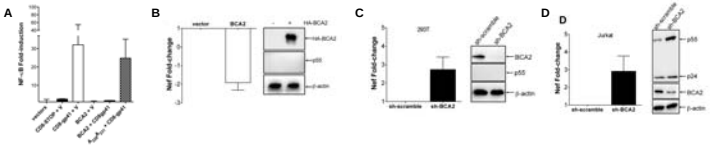
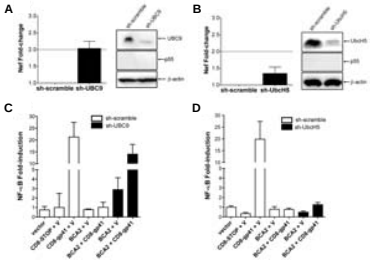


Figure 4. BCA2 prevents HIV-1-dependent activation of NF-κB and restricts HIV-1 transcription

To investigate whether BCA2 outcompetes HIV-1 gp41 in the regulation of NF-κB, additional luciferase assays were performed. Whereas CD8-gp41 led to more than 30-fold induction in NF-κB activity in the absence of HA-BCA2, co-expression of CD8-gp41 and HA-BCA2 resulted in the complete inhibition of this cascade. However, co-expression of the BCA2 RING-mutant and CD8-gp41 had no impact on the ability of gp41 to induce this pathway (Fig. 4A). To assess if this effect detrimentally affects HIV-1 transcription, we analyzed the expression of *nef* in HA-BCA2 expressing 293T cells transfected with HIV-1 NL4.3 proviral DNA at 6 hours post-transfection. Remarkably, cells overexpressing HA-BCA2 showed a 1.92-fold reduction in *nef* expression (Fig. 4B). By contrast, BCA2 depletion led to a 2.8- and 2.91-fold increase in *nef* expression in 293T and CD4+T cells, respectively (Fig. 4C-4D). **Therefore, the BCA2-dependent suppression of NF-κB significantly reduces HIV-1 transcriptional capacity.**

Figure 5. BCA2 requires UBC9 but not UbcH5 to inhibit NF-κB and restrict HIV-1 transcription

UBC9 and UbcH5 are the two known E2-conjugating enzymes that interact with BCA2. To assess whether UBC9 or UbcH5 are required for BCA2-mediated inhibition of NF-κB, RT-qPCR assays were performed in UBC9- and UbcH5-depleted cells. *Nef* expression increased 2-fold in UBC9-knocked down 293T cells (Fig. 5A). However, in UbcH5-depleted cells there was only a modest 1.34-fold increase in *nef* transcription (Fig. 5B). Furthermore, in UBC9-depleted cells co-expressing HA-BCA2 along with CD8-gp41, the NF-κB activity was restored (Fig. 5C). By contrast, no effects on the BCA2-dependent inhibition of NF-κB were observed under conditions of UbcH5-depletion (Fig. 5D). **Hence, BCA2 requires UBC9 to efficiently inhibit NF-κB.**



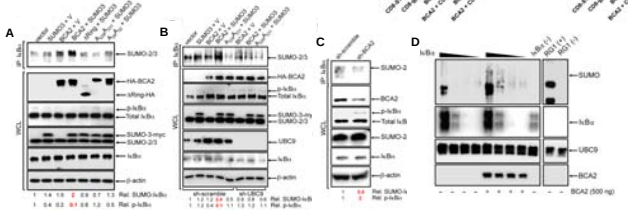


Figure 6. BCA2 inhibits NF-κB responses by enhancing the SUMOylation of IκBα

UBC9 regulates NF-κB by SUMOylating IκBα. To examine if BCA2 functions as an E3 SUMO-ligase in this process, IκBα's post-translational modifications (phosphorylation vs. SUMOylation) were investigated. The expression of HA-BCA2 alone or in combination with SUMO-3-myc resulted in a 2-fold increase in SUMO-IκBα. By contrast, the SUMO-IκBα levels were reduced in the presence of BCA2 RING-finger mutants (Fig. 6A). Depletion of UBC9 in HA-BCA2+ cells led to a 3-fold decrease in SUMO-IκBα and a ~10-fold increase in p-IκBα (Fig. 6B). Similarly, knocking down BCA2 led to a 1.6-fold reduction in SUMO-IκBα and a 2-fold increase in p-IκBα (Fig. 6C). To confirm that BCA2 enhances IκBα SUMOylation, an *in vitro* SUMOylation assay was conducted with decreasing concentrations of IκBα and constant amounts of BCA2 and UBC9. Remarkably, the rate of SUMOylation increased in the presence of BCA2 (Fig. 6D)**. In conclusion, BCA2 serves as an E3 SUMO-ligase in the SUMOylation of IκBα.**

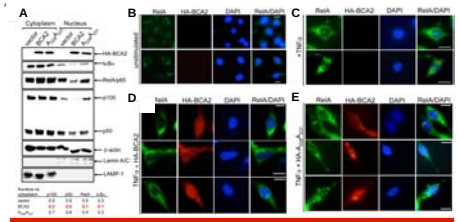
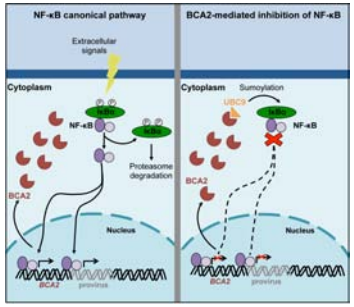


Figure 7. BCA2 prevents the nuclear translocation of NF-κB

To confirm that the BCA2-promoted SUMOylation of IκBα further prevents the nuclear translocation of NF-κB, the cytoplasmic vs. nuclear levels of NF-κB, BCA2 and IκBα were measured. The nuclear levels of RELA/p65, p100/p50 and IκBα were significantly reduced in cells expressing HA-BCA2 (Fig. 7A). However, the nuclear levels of NF-κB and IκBα remained unchanged in the presence of an empty vector or a BCA2 RING-defective mutant. These results were further confirmed with cellular images (Fig. 7B-7E). **Therefore, BCA2 prevents the nuclear translocation of NF-κB.**

CONCLUSIONS



- **BCA2 is induced by NF-κB pro-inflammatory cytokines and up-regulation of BCA2 provides a regulatory negative feedback on NF-κB.**
- **BCA2 serves as an E3 SUMO-ligase in the SUMOylation of IκBα, a previously unrecognized activity of BCA2.**
- **The BCA2-mediated SUMOylation of IκBα enhances the sequestration of NF-κB components in the cytoplasm.**
- **The BCA2-mediated inhibition of the NF-κB pathway impairs HIV-1 transcriptional capacity and replication.**

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