I κ B α inhibits apoptosis at the outer mitochondrial membrane independently of NF- κ B retention

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Abstract

I κ B α resides in the cytosol where it retains the inducible transcription factor NF- κ B. We show that I κ B α also localises to the outer mitochondrial membrane (OMM) to inhibit apoptosis. This effect is especially pronounced in tumour cells with constitutively active NF- κ B that accumulate high amounts of mitochondrial I κ B α as a NF- κ B target gene. 3T3 I κ B α ^{-/-} cells also become protected from apoptosis when I κ B α is specifically reconstituted at the OMM. Using various I κ B α mutants, we demonstrate that apoptosis inhibition and NF- κ B inhibition can be functionally and structurally separated. At mitochondria, I κ B α stabilises the complex of VDAC1 and hexokinase II (HKII), thereby preventing Bax recruitment to VDAC1 and the release of cytochrome *c* for apoptosis induction. When I κ B α is reduced in tumour cells with constitutively active NF- κ B, they show an enhanced response to anticancer treatment in an *in vivo* xenograft tumour model. Our results reveal the unexpected activity of I κ B α in guarding the integrity of the OMM against apoptosis induction and open possibilities for more specific interference in tumours with deregulated NF- κ B.

Keywords apoptosis; cell death; I κ B α ; mitochondria; VDAC1

Subject Categories Autophagy & Cell Death; Cancer

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Introduction

I κ B α functions as inhibitor of the inducible transcription factor NF- κ B by sequestering its subunits in the cytosol (Baeuerle & Baltimore, 1996). For the activation of NF- κ B, I κ B α is phosphorylated, ubiquitinated and degraded by the proteasome (Hayden & Ghosh, 2008). This allows the remaining dimeric subunits,

composed of relA (p65), c-rel, relB, p50 or p52, to enter the nucleus, bind promoters of a wide range of target genes and initiate their transcription. In most cells, the activation of the NF- κ B target genes implements a state of cell death resistance (Luo *et al*, 2005). As a target gene, I κ B α is upregulated by NF- κ B and inactivates the transcription factor in a feedback loop (Le Bail *et al*, 1993). In this capacity, I κ B α exerts a pro-apoptotic activity as it inhibits the anti-apoptotic NF- κ B. Whether I κ B α has, besides NF- κ B inhibition, additional activities and whether it directly contributes to apoptosis inhibition as a NF- κ B target gene are unknown.

The mitochondrial outer membrane permeabilisation (MOMP) is acknowledged to be a pivotal event in the signalling for apoptosis (Spierings *et al*, 2005). It leads to the release of pro-apoptotic factors such as cytochrome *c* that can then assemble the apoptosome, a caspase-activation platform (Jiang & Wang, 2004). MOMP is accomplished by Bcl-2 family members, in particular Bax and Bak, that form pores in the OMM. This activity must be tightly controlled; however, the underlying mechanism of how this is accomplished is only incompletely understood. VDAC1 is a protein at the OMM that can recruit cytosolic Bax for MOMP. This, in turn, is abrogated by hexokinase II (HKII), which can bind to VDAC1 and prevent the interaction with Bax (Pastorino & Hoek, 2008).

In this study, we uncovered a novel and unexpected apoptosis function of I κ B α . We found that it localises to the OMM where it interacts with VDAC and HKII to stabilise this complex and prevent Bax-mediated cytochrome *c* release for apoptosis. A range of experiments including the use of specific I κ B α mutants with different sub-cellular localisations or distinct domains, I κ B α variants with opposing effects on NF- κ B and conditions without or with constitutive NF- κ B activity indicate that the novel anti-apoptotic activity of I κ B α at the OMM can be separated from its known ability to inhibit NF- κ B. When I κ B α is reduced by RNAi in tumour cells with constitutively active NF- κ B, it leads to a re-sensitisation for apoptosis induction *in vitro* and *in vivo*.

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Results

I κ B α localises to the outer mitochondrial membrane

In order to explore the sub-cellular residence of I κ B α , we used confocal immunofluorescence microscopy and determined that in HeLa, MCF7 and 3T3 cells I κ B α partly co-localised with mitochondria, while in the cell lines MDA-MB-231, PC3 and HCT-116 that display constitutive NF- κ B activity (Nakshatri *et al*, 1997; Dejardin *et al*, 1999; Suh *et al*, 2002; Sakamoto *et al*, 2009), we observed an almost complete spatial overlap (Fig 1A, Supplementary Fig S1A). Treatment of intact mitochondria isolated from HeLa cells with proteinase K revealed that the enzyme gained access to I κ B α as it could substantially reduce the I κ B α signal in a protein blot and likewise that of the OMM protein Tom20 as a control, while the inner mitochondrial protein cytochrome *c* was protected (Fig 1B). With isolated mitochondria from the other cell lines, we likewise found that I κ B α could be cleaved by proteinase K, suggesting that it was associated with the OMM (Fig 1C). Using the same fractionating protocol from Fig 1B, we observed a pronounced accumulation of I κ B α in the mitochondrial fraction of PC3 cells and a more equal distribution between the cytosolic and mitochondrial fraction in MCF7 cells (Supplementary Fig S1B). The co-localisation of I κ B α in PC3 cells was predominantly to mitochondrial VDAC1 when compared with an ER marker (Supplementary Fig S1C) and could be observed in HCT-116 and MDA-MD 231 cells as well and to a lesser degree also in primary HUVEC cells (Supplementary Fig S1D). Mitochondrial I κ B α was still susceptible to degradation upon TNF stimulation of the cells (Supplementary Fig S1E).

Reduction of I κ B α changes the cells' apoptosis sensitivity dependent on its presence at mitochondria

NF- κ B is known to be constitutively active in many malignant cells, which would lead to an upregulation of its target gene I κ B α . Using Oncomine, we indeed found that I κ B α is increased in many microarray studies on tumours when compared to normal cells (Supplementary Fig S2A). Our experiments revealed that in MDA-MB-231 and PC3 cells, which harbour prominent total I κ B α levels and constitutive NF- κ B, its protein accumulated at mitochondria (Fig 2A, Supplementary Fig S2B). In HCT-116 cells that are also NF- κ B-positive, I κ B α likewise showed a noticeable concentration at mitochondria, even though its total I κ B α was less pronounced. In order to further investigate the role of I κ B α in those cells, we stably knocked down I κ B α in MDA-MB-231 cells using lentiviruses. Constitutively active NF- κ B remained unaffected, indicating that it is activated by a process other than I κ B α degradation in these cancer cells (Prasad *et al*, 2009) (Fig 2B and C). We tested a number of target genes and in line with the unaltered NF- κ B binding activity their expression levels did not change (Supplementary Fig S2C).

The treatment with various apoptosis inducers revealed that I κ B α reduction rendered these cells significantly more sensitive to apoptosis (Fig 2D, Supplementary Fig S2D and E). When I κ B α was targeted in PC3 cells, which likewise display constitutively active NF- κ B and a high amount of I κ B α at mitochondria (Fig 2A), the cells similarly became more sensitive to apoptosis induction than when transduced with a scrambled shRNA sequence (Fig 2E–G, Supplementary Fig S2D and F). Annexin V staining revealed a good

correlation with DiOC₆/PI staining in the MDA-MD 231 and PC3 cells (Supplementary Fig S2G, upper panels). The same effect could be observed at additional time points (Supplementary Fig S2H). We also used an additional RNAi construct that was less efficient in targeting I κ B α and still sensitised the cells for apoptosis (Supplementary Fig S2I).

To also explore the role of I κ B α at the OMM for apoptosis regulation in cells with inducible NF- κ B and low mitochondrial I κ B α , we resorted to 3T3 cells. We engineered a construct with I κ B α fused to β -actin (I κ B α - β -actin) to retain it in the cytosol and another chimera, using a N-terminal I κ B α fusion to the mitochondrial localisation sequences of mitoNEET (Wiley *et al*, 2007), to specifically target it to the OMM (Supplementary Fig S3A). We then reconstituted I κ B α ^{-/-} 3T3 cells with the I κ B α variants and with I κ B α -wt (Supplementary Fig S4A). Diverse signals for apoptosis such as ionomycin and etoposide as well as ANT1 (adenosine nucleotide translocator-1) transfection caused enhanced apoptosis in the I κ B α - β -actin-reconstituted cells in comparison with the I κ B α ^{-/-} cells, most likely through inhibition of NF- κ B and the consequent reduced transcription of anti-apoptotic NF- κ B target genes (Fig 2H, Supplementary Fig S4B). Reconstitution with MEET-I κ B α , on the other hand, not only overcame this apoptosis sensitisation, but led to a decrease in apoptosis (Fig 2H). NF- κ B binding was inhibited to the same extent in the MEET-I κ B α - and I κ B α - β -actin-reconstituted cells allowing comparison of their sensitivities to cell death independently of differences in NF- κ B target gene activation (Supplementary Fig S3B). Moreover, upon transient transfection, only the mitochondrial MEET-I κ B α but not I κ B α - β -actin could reduce apoptosis (Supplementary Fig S4C). The reconstitution of the I κ B α ^{-/-} cells with WT I κ B α led to an intermediate, but still significant apoptosis reduction for most signals (Fig 2H). We confirmed this with additional cell death stimuli (Supplementary Fig S4D). To directly compare cellular systems with constitutively active versus inducible NF- κ B leading to normal or increased mitochondrial I κ B α , respectively, we quantified apoptosis by serum starvation when I κ B α was reduced in 3T3, PC3 and MDA-MB-231 cells. In accordance with our expectation, we observed a stronger apoptosis sensitisation in the cells with constitutive NF- κ B and mitochondrial I κ B α accumulation (Fig 2I, Supplementary Fig S4E). These results were supported by additional experiments: In cells with prominent mitochondrial I κ B α localisation such as HCT-116, a partial I κ B α reduction by RNAi was sufficient to cause a significant sensitisation for apoptosis by ANT1 and t-Bid (Fig 2A, Supplementary Fig S4F). On the other hand, targeting I κ B β did not lead to a change in apoptosis sensitivity (Supplementary Fig S4G).

I κ B α at the outer mitochondrial membrane inhibits apoptosis

In order to investigate the effects of mitochondrial I κ B α accumulation, we targeted I κ B α to the OMM and to the mitochondrial intermembrane space, respectively, using a N-terminal I κ B α fusion to the mitochondrial localisation sequence of Smac/DIABLO (S/D) (Ozawa *et al*, 2007) and the above-mentioned mitoNEET chimera (Fig 3A). Immunofluorescence confirmed the localisation of the fusion constructs to mitochondria and proteinase K digest on isolated mitochondria in combination with Western blotting supported their residence in the expected compartments (Supplementary Fig S5A). With the OMM playing a pivotal role in

Figure 1. IkBα is found at the outer mitochondria membrane.

A Confocal photomicrographs at 63× of IkBα distribution in several cells lines.
B Mitochondrial and cytosolic fractions were isolated from HeLa cells and treated with proteinase K to identify the sub-compartmental localisation of IkBα (*, unspecific band).
C Mitochondria fractions of the indicated cell lines were treated with proteinase K to validate the localisation of IkBα within the organelle. The same conditions and mitochondria purity were used as in (B).

Source data are available online for this figure.

Figure 2.

Figure 2. Effect of I κ B α reduction on apoptosis sensitivity.

- A Whole-cell lysates and mitochondria isolated from the indicated cell lines were probed for the levels of I κ B α protein and the indicated proteins in a Western blot. VDAC1 and actin were used as loading controls. 50 μ g of protein were loaded for both whole-cell lysate and mitochondria.
- B, C I κ B α expression levels were reduced in MDA-MB-231 cells by viral shRNA (shI κ B α), and the effect on NF- κ B activation was probed by Western blot (*, unlabelled oligo; SC, scrambled control).
- D I κ B α knock-down and parental cells from (B, C) were subjected to treatment with clotrimazole (20 μ M) (left panel), arsenic trioxide (10 μ M) (middle panel) and doxorubicin (2 μ M) (right panel), and apoptosis was quantified after 24 h by FACS.
- E, F I κ B α was knocked down in PC3 cells, and the status of NF- κ B was probed by Western blot (*, unlabelled oligo).
- G PC3 cells from (E, F) were treated with docetaxel (5 nM) (left panel), staurosporine (1 μ M) (middle panel) and actinomycin D (1.4 μ M) (right panel), and apoptosis was quantified by FACS.
- H 3T3 I κ B α ^{-/-} cells were reconstituted using lentiviral vectors coding for I κ B α -wt, MEET-I κ B α and I κ B α fused with beta-actin. Cells were treated as described in Materials and Methods, and apoptosis was quantified 24 h post-treatment by FACS. The effect of the reconstitution on apoptosis was compared under conditions of non-active NF- κ B (in grey) or compared to cells reconstituted with an empty vector (EV; in black).
- I 3T3 WT, 3T3 I κ B α ^{-/-}, PC3/sc, PC3/shI κ B α , MDA-MB-231/sc and MDA-MB-231/shI κ B α cells were grown without serum for 72 h, and apoptosis was quantified through CaspaGlo for caspase-3/7 (Promega).

Data information: Data in (D, G–I) were obtained from $n > 3$ experiments; values in graphs represent means $\pm$ s.d.; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Source data are available online for this figure.

apoptosis induction, we tested the activity of the I κ B α fusion constructs in an apoptosis assay. MitoNEET-I κ B α (MEET-I κ B α) completely abolished apoptosis induced by tBid and ANT1 transfection. Bax and VDAC1 were likewise efficiently inhibited by MEET-I κ B α , while apoptosis induced by caspase-8 was only weakly affected (Fig 3B). WT I κ B α was also an efficient apoptosis inhibitor (Fig 3B), while the S/D-I κ B α fusion and I κ B β , which differs mainly in its N-terminus from I κ B α , had no effect on apoptosis by pro-apoptotic genes (Fig 3C and D). ANT1 expression caused apoptosis in those cells as indicated by PARP cleavage and its inhibition by Bcl-XL (Supplementary Fig S2G, lower panel). A fusion of I κ B α to the mitochondrial localisation sequence of yeast mcr1, which is associated with the OMM (Hahne *et al*, 1994), also led to inhibition of ANT1-induced apoptosis in 293T, MCF7 and HeLa cells (Supplementary Fig S5B–D). Also, serum starvation-induced activation of caspases was efficiently inhibited by WT I κ B α and MEET-I κ B α (Supplementary Fig S5E). To determine the relation of apoptosis and NF- κ B inhibition, a non-degradable form of I κ B α (I κ B α -SR) and an I κ B α mutant devoid of NF- κ B inhibition (I κ B α -110A3) (Sachdev *et al*, 1998) were co-transfected with ANT1 and Bax and found to be as effective as WT I κ B α for apoptosis inhibition (Fig 4A). Both I κ B α constructs could be detected associated with mitochondria (Supplementary Fig S6A). A reporter assay confirmed that under these conditions, the endogenous NF- κ B system was not activated (Fig 4B). The two I κ B α constructs as well as the WT I κ B α were also active against apoptosis induced by genotoxic drugs (Supplementary Fig S6B). In order to explore whether the effect on apoptosis could structurally be separated from the known activity of I κ B α to inhibit NF- κ B, we generated MEET-I κ B α deletion mutants including one of the I κ B α N-terminus and one of its C-terminus that are dispensable and required for NF- κ B inhibition, respectively (Hatada *et al*, 1993). An inverse structural requirement was found for apoptosis inhibition: the N-terminus of I κ B α was necessary for apoptosis inhibition, while the C-terminus could be deleted without compromising apoptosis repression by I κ B α (Fig 4C, top and lower left panel). The fusion to mitoNEET sequences ensured that all deletion mutants were localised to mitochondria (Supplementary Fig S6C). To determine the minimal I κ B α domains sufficient for apoptosis inhibition, we generated additional mutants

(Supplementary Fig S6D) and found that a construct encompassing the N-terminal residues 1–109 was able to diminish apoptosis (Fig 4C, top and lower right panel). Apoptosis repression was also observed with the I κ B α 1–109 construct without the mitoNEET moiety (Supplementary Fig S6E and F). Altogether these experiments strongly suggested that the effects on apoptosis exerted by I κ B α were independent of NF- κ B inhibition.

I κ B α stabilises the VDAC–hexokinase II complex via its interaction with VDAC1

In an effort to explore the function of I κ B α at mitochondria, we tried to identify its interaction partners at this organelle. Using immunoprecipitations, we found that I κ B α interacted, directly or indirectly, with several mitochondrial proteins such as ANT1 and CK1 located in the IMM and the intermembrane space, respectively, and also with VDAC1 at the OMM (Fig 5A). No interaction was detected with TOM20 and cytochrome *c*. Since VDAC1 localises to the OMM and as I κ B α exerts its anti-apoptotic effect at the same locale, it constituted a relevant target. Accordingly, I κ B β , which was found to be impaired in apoptosis inhibition (Fig 3E), was not able to interact with VDAC1 (Supplementary Fig S7A). Moreover, all I κ B α mutants that were active for apoptosis repression (Fig 4C), also associated with VDAC, while the mutant lacking the N-terminus only, showed reduced binding (Fig 5B). We also observed an interaction with VDAC1 for WT I κ B α and its mutant 1–109 [which is still able to inhibit apoptosis (Supplementary Fig S6F)] without the mitoNEET moiety (Supplementary Fig S7B). ShRNA-mediated knock-down of VDAC1 by two independent RNAi constructs, but not VDAC3, reduced the potential of MEET-I κ B α to inhibit apoptosis (Fig 5C and D, Supplementary Fig S7C) and completely abrogated its activity in reconstituted 3T3 cells (Fig 5E). The interaction with VDAC1 appears to be lost upon apoptosis induction before any biochemical features of apoptosis were observed as confirmed by DiOC₆/PI staining (Fig 5F, Supplementary Fig S7D). These experiments suggested that I κ B α targets VDAC1 to inhibit apoptosis. As VDAC1 is known to recruit the pro-apoptotic Bcl-2 family member Bax, which causes MOMP and apoptosis induction (Shimizu *et al*, 1999), we investigated a possible functional interaction. We co-incubated

Figure 3. I κ B α inhibits apoptosis at the OMM.

A I κ B α was fused with the mitochondria localisation sequences of Smac-DIABLO (S/D) and mitoNEET for targeting to the intermembrane space (IMS) and to the outer mitochondrial membrane (OMM), respectively. The fusion proteins are depicted schematically.

B 293T cells were transfected with the indicated apoptosis inducers together with constructs for β -gal, WT I κ B α or MEET-I κ B α at a ratio of 1:2, and apoptosis was quantified by FACS 24 h post-transfection.

C 293T cells were transfected with β -gal or the S/D-I κ B α construct together with the indicated apoptosis-inducing genes, and apoptosis was quantified by DiOC₆/PI after 24 h.

D Effect of I κ B β on apoptosis inhibition. Expression plasmids for β -gal or I κ B β were co-transfected with the indicated apoptosis-inducing genes at a ratio of 1:1 into 293T cells. After 24 h, apoptosis was quantified by DiOC/PI staining and flow cytometry.

Data information: Data in (B–D) were obtained from $n > 3$ experiments; values in graphs represent means $\pm$ s.d.; * $P < 0.05$ *** $P < 0.001$. Source data are available online for this figure.

isolated mitochondria with or without *in vitro*-translated I κ B α and added increasing amounts of recombinant Bax. The release of cytochrome *c* served as a read-out for the permeabilisation of the OMM as a pivotal step in apoptosis induction. Upon addition of I κ B α , the release of cytochrome *c* was completely abrogated at all concentrations of Bax used (Fig 6A). In PC3 cells, the presence of I κ B α caused a considerably delayed cytochrome *c* release and less efficient Bax integration than in the absence of I κ B α when apoptosis was induced by staurosporine (Fig 6B). HKII is known to interact with VDAC1 at the OMM, which inhibits the recruitment of Bax to this protein complex (Pastorino *et al*, 2002). Upon apoptosis induction, HKII is released from VDAC1 and Bax associates with VDAC1 to form a pore in the OMM (Pastorino *et al*, 2005). Immunofluorescence revealed a spatial overlap of HKII, VDAC1 and I κ B α (Supplementary Fig S7E), and we also observed the co-localisation of VDAC1 with I κ B α in various cell lines including primary HUVEC cells (Supplementary Fig S1D). The interaction of endogenous HKII and I κ B α was detected via co-immunoprecipitation (Fig 6C). Mapping of the interaction revealed that residues 1–70 of I κ B α were dispensable and that the N-terminal 1–109

residues interacted with HKII (Fig 6D). Reducing HKII in the cells rendered WT I κ B α and MEET-I κ B α unable to inhibit ANT1- and ionomycin-induced apoptosis (Supplementary Fig S7F). The reduction of either VDAC1 or HKII had no effect on the association of I κ B α with the remaining complex partner (Supplementary Fig S7G). With VDAC1 being associated with the outer mitochondrial membrane, this indicates that HKII is dispensable for its mitochondrial targeting. Mitochondria from I κ B α - or control vector-expressing HeLa cells were isolated and assayed for the interaction between VDAC1 and HKII upon addition of increasing concentrations of the HKII-release peptide HXK2VBD, which comprises the first N-terminal residues of HKII. The association between VDAC1 and HKII was undisturbed in the presence of I κ B α , while it was almost completely abolished in its absence (Fig 6E). Conversely, when we increased the I κ B α level in non-apoptotic cells, we observed a stronger interaction between HKII and VDAC1 (Supplementary Fig S7H). When we used clotrimazole to disrupt the VDAC1–HKII association in I κ B α ^{−/−}-deficient and MEET-I κ B α -reconstituted cells (Fig 6F, top panels) as well as in HeLa cells transfected with WT I κ B α (Fig 6G), we likewise

Figure 4. I κ B α inhibits apoptosis independently of NF- κ B inhibition.

A Constructs for WT I κ B α , a non-degradable form of I κ B α (I κ B α SR), and an I κ B α mutant that is impaired in inhibiting NF- κ B (I κ B α -110A3) were compared for their ability to suppress ANT1- and Bax-induced apoptosis.

B A reporter activity assay for NF- κ B was conducted upon transfection of β-gal, Tax, Bax or ANT1 into 293T cells.

C The fusion construct for MEET-I κ B α or the indicated deletion mutants (schematically depicted on the top) were co-transfected into 293T cells together with ANT1 at a ratio of 1:2 and apoptosis quantified by FACS after 24 h (left). Fusion constructs comprising the indicated sequences were co-transfected with ANT1 at a ratio of 1:2, and apoptosis was quantified after 24 h (right).

Data information: Data were obtained from $n > 3$ experiments; values in graphs represent means $\pm$ s.d.; *** $P < 0.001$.

Source data are available online for this figure.

observed an increased stabilisation of the VDAC1–HKII interaction in the presence of I κ B α . A co-IP revealed that the interaction between VDAC1 and HKII was reduced by clotrimazole in the absence of I κ B α , while this effect was prevented in the presence of MEET-I κ B α (lower panels Fig 6F).

I κ B α knock-down in cancer cells with constitutive active NF- κ B sensitises to anticancer compounds *in vivo*

MDA-MB-231 cells that display constitutively active NF- κ B and prominent I κ B α accumulation at mitochondria (Fig 2A) were

Figure 5. Mitochondrial IκBα targets VDAC1 for apoptosis inhibition.

- A** HCT-116 cells were lysed in RIPA buffer and the endogenous IκBα subjected to immunoprecipitation. Membranes were probed for the indicated mitochondrial proteins. Control (Ctrl) was the IgG corresponding to the IP antibody.
- B** 293T cells were transfected with the indicated deletion mutants of IκBα and their interaction probed in a co-IP with endogenous VDAC1. Arrows indicate the expected sizes of the constructs.
- C, D** VDAC1 was stably reduced by two shRNAs and DiOC₆/PI was measured by FACS in β-gal-, MEET-IκBα-, WT-IκBα- or S/D-IκBα-expressing 293T cells upon co-transfection of an expression vector for ANT1 (**C**) or treatment with ionomycin (**D**). Knock-down level of VDAC1 in 293T cells used for these experiments is shown in a Western blot.
- E** 3T3 IκBα-reconstituted cells (from Fig 4) were transduced with lentiviruses to knock down VDAC1. A virus carrying a scrambled sequence served as a control (scramble). Two weeks post-selection, the cells were treated with either ionomycin (10 μM) or clotrimazole (40 μM), and apoptosis was quantified 4 h later.
- F** HeLa (left) and HCT-116 (right) cells were treated with As₂O₃; 10 h after As₂O₃ administration, IκBα was immunoprecipitated and VDAC1 was probed by Western blot. Control was the IgG corresponding to the IP antibody.

Data information: Data in (C–E) were obtained from $n > 3$ experiments; values in graphs represent means $\pm$ s.d.; *** $P < 0.001$.

Source data are available online for this figure.

Figure 6.

engineered to stably express shI κ B α or scrambled constructs (Fig 2B). They were then bilaterally engrafted in nude mice, which were treated with PBS or doxorubicin for 5 weeks, and tumour size measurements were taken twice weekly. Tumours with reduced I κ B α decreased in volume upon drug treatment, while the control cells were much less affected (Fig 7, Supplementary Fig S8A).

Discussion

We have shown here that, unexpectedly, I κ B α , besides its inhibitory effect on NF- κ B, also guards the integrity of the OMM and thereby controls the intrinsic, mitochondrial pathway for apoptosis. This novel activity of I κ B α is especially pronounced in cells that

Figure 6. Mitochondrial I κ B α inhibits HKII release from VDAC1- and Bax-induced cytochrome c release.

- A *In vitro*-translated β -gal or I κ B α were added to mitochondria from 293T cells, which were then treated with increasing concentrations of recombinant Bax, and the release of cytochrome c in the supernatant was assessed. The — signs on the top of the Western blot indicate the use of β -gal.
- B I κ B α was downregulated by shRNA in PC3 cells, and apoptosis was induced by staurosporine (STS). At different time points, cells were harvested, subcellular fractions isolated, and mitochondria probed for the reduction of I κ B α and, after an alkaline wash, for the recruitment of Bax in a Western blot. Mitochondrial HSP60 was used as a loading control. The cytosolic fractions were investigated for the release of cytochrome c.
- C I κ B α interacts with HKII. PC3 cells were lysed in RIPA buffer and the interaction of endogenous I κ B α probed in a co-IP with endogenous HKII. Membranes were probed for the indicated mitochondrial proteins. Control (Ctrl) was the IgG corresponding to the IP antibody.
- D Mapping the interaction between I κ B α and HKII. The indicated FLAG-tagged I κ B α mutants were transfected in 293T cells. Immunoprecipitations against FLAG were performed 24 h post-transfection and the immunoprecipitates separated in a 4–15% gel (Bio-Rad) to determine the domain of I κ B α that is sufficient to interact with HKII. Expression of the mutants is shown in the Western blot using 1% of the total lysate.
- E Mitochondria from control- or I κ B α -transfected HeLa cells were treated with the HKII-release peptide HXK2VBD, and the interaction between HKII and VDAC1 was monitored with immunoprecipitations.
- F Mitochondria isolated from I κ B α ^{−/−} or MEET-I κ B α -reconstituted 3T3 cells were treated with clotrimazole (CTZ; 80 μ M, 30 min), and the indicated proteins were detected associated with mitochondria (top panels). Under the same conditions, immunoprecipitations were performed against VDAC and the indicated interacting proteins detected (bottom panels). NT, no treatment.
- G Transfection of I κ B α in HeLa cells stabilises the HKII–VDAC interaction. HeLa cells transfected with WT I κ B α (right) or a control vector (left) were subjected to the indicated immunoprecipitations after treatment with the indicated concentrations of clotrimazole (CTZ). Percentages of the HKII signals compared with the untreated sample are indicated below the blots.

Source data are available online for this figure.

accumulated I κ B α at mitochondria such as tumour cells with constitutive NF- κ B activity (Fig 2B–G). In cells with baseline mitochondrial I κ B α , the protective effect was revealed only upon complete knock-down of I κ B α (Fig 2H and Supplementary Fig S4D), probably as it was otherwise obscured by its known activity to inhibit the anti-apoptotic transcription factor complex NF- κ B.

I κ B α 's ability to regulate MOMP and apoptosis is accomplished by stabilising the complex of HKII and VDAC1, thereby inhibiting Bax-mediated cytochrome c release (Fig 6). HKII is known to bind to VDAC1, the most abundant VDAC isoform, via its N-terminus and its putative BH4 domain (Pastorino & Hoek, 2008). This seems to favour the oligomeric association of VDAC proteins and keeps these proteins unable to interact with Bax for apoptosis induction. It is well established that the pro-apoptotic multi-domain members of the Bcl-2 family form proteinaceous or, indirectly, lipidic channels in the OMM as a critical step in apoptosis induction (Shamas-Din *et al*, 2013). The observed importance for VDAC for Bax-induced apoptosis could be explained by VDAC acting as a recruitment or interaction partner for the latter, similar to the described Bak–VDAC2 complex (Roy *et al*, 2009). With the novel activity at the OMM described here, I κ B α exerts the same effect as the enterobacterial fimA protein, which likewise stabilises the VDAC–HKII interaction and inhibits apoptosis (Sukumaran *et al*, 2010). Our results indicate that I κ B α can make contact with VDAC1 and HKII independently of each other and hence might act like a molecular clamp for this complex (Supplementary Fig S8B).

The role of VDAC in apoptosis has been questioned in one study that used VDAC1- and VDAC3-deficient and VDAC2 knock-down cells (Baines *et al*, 2007). However, VDAC proteins are proven modulators in apoptosis signalling and individual members control several apoptosis cascades through mitochondria (Shoshan-Barmatz *et al*, 2006, 2009; Simamura *et al*, 2008; Abu-Hamad *et al*, 2009; Tomasello *et al*, 2009; McCommis & Baines, 2012). Our discovery could explain previous reports according to which I κ B α expression is anti-apoptotic, which, however, was not specifically addressed by the authors (Vannucchi *et al*, 2005).

The interaction of I κ B α with the VDAC1–HKII complex is specific: I κ B β is not able to associate with VDAC1 and inhibit

apoptosis (Fig 3D, Supplementary Fig S7A). We have also found cell lines with constitutively active NF- κ B that harboured similar I κ B α levels in whole-cell lysates but dramatically differential I κ B α association with the OMM (Figs 1A and 2A). The localisation of NF- κ B subunits to mitochondria was revealed previously (Bottero *et al*, 2001; Cogswell *et al*, 2003), and in particular, Bottero *et al* also found the association with the OMM using immunoelectron microscopy and proteinase K digest but its role for apoptosis regulation was not investigated.

I κ B α 's role for apoptosis inhibition at the OMM is spatially and functionally separable from its well-known function to inhibit the transcription factor NF- κ B (Fig 4). Our mapping experiments indicated that, in contrast to the inhibition of NF- κ B, the N-terminus of I κ B α is responsible for its repression of apoptosis (Fig 4C). Moreover, specific I κ B α mutants with either reduced or constitutive NF- κ B retention (Fig 4A), and I κ B α variants with differential localisation (Supplementary Fig S3) allowed separate the pro- and the anti-apoptotic activity of I κ B α . Finally, experiments under conditions with or without constitutive NF- κ B activity (Figs 2 and 4B) indicated that the novel activity of I κ B α to repress apoptosis at the OMM is distinct from its known ability to inhibit NF- κ B.

In most cell types, the activation of the transcription factor NF- κ B leads to an anti-apoptotic response through the upregulation of cell death repressors. Hence, as the inhibitor of NF- κ B, I κ B α exerts a pro-apoptotic function, which is counteracted by its novel and unexpected activity to guard the integrity of the OMM against apoptosis induction as presented here. Therefore, during its degradation upon activation of NF- κ B, while it is re-synthesised as a consequence of the transcriptional activity of NF- κ B, I κ B α contributes to the balance of survival and cell death signals at all time points in the NF- κ B activation cycle. This is especially important, as many of the signals that activate NF- κ B constitute cell stress. We have observed an effect on apoptosis in cells with inducible NF- κ B only when I κ B α was knocked out. Partial reduction by knock-down did not show this effect (Supplementary Fig S4D and F).

I κ B α 's activity to inhibit apoptosis at the OMM is spatially and functionally separable from its function to inhibit the transcription

Figure 7. Effect of I κ B α inhibition on apoptosis in tumour cells with constitutively active NF- κ B *in vitro* and *in vivo*.

Average tumour volume was calculated as described in Materials and Methods. The mice were treated over a period of 5 weeks and injections with either PBS or doxorubicin (6 mg/kg once a week *i.v.*) were done at the time points designated with A each week. Representative pictures of tumours excised from mice (right panels). Data were obtained from $n > 3$ experiments; values in graphs represent means $\pm$ s.d.

Source data are available online for this figure.

factor NF- κ B, even though in normal 3T3 cells mitochondria-targeted I κ B α exerts both effects (Fig 2H). In this scenario, mitochondrial I κ B α affects mitochondria and NF- κ B at the same time as it can establish the relevant interactions simultaneously.

In numerous tumours, on the other hand, I κ B α is rendered unable to inhibit NF- κ B based on a number of heterogeneous mutations (Sethi *et al*, 2008). As a consequence, NF- κ B is constitutively active and this is required for the survival of the malignant cells (Prasad *et al*, 2009). Many studies such as Shukla *et al* detected that advanced, more aggressive tumour stages show an increase of active NF- κ B together with an increased accumulation of I κ B α as its target gene (Shukla *et al*, 2004). We have focused here on such tumour cells that are NF- κ B-positive and nevertheless express (NF- κ B inhibition-inactive) I κ B α , features that have been found in many malignant cancers (Sethi *et al*, 2008). Under such conditions, NF- κ B is not available for I κ B α as a binding partner. Because of this and since I κ B α is a target gene of NF- κ B, its protein will accumulate and interact with VDAC at mitochondria to inhibit apoptosis (Supplementary Fig S8B). In cells with inducible NF- κ B, I κ B α has the dual activity of stabilising VDAC1–HKII and masking the localisation sequence of RelA. I κ B α can perform these two activities simultaneously due to different domains that facilitate this. Nevertheless, in such cells, the mitochondrial accumulation of I κ B α is reduced as its interaction partner RelA is known to associate with the cytoskeleton forming a cytosolic anchor for I κ B α (Are *et al*, 2000). Of note, the C-terminus of I κ B α is mutated in Hodgkin's lymphoma, while its N-terminus remains intact (Cabannes *et al* 1999). This disturbs only

its effect on retaining NF- κ B and preserves its anti-apoptotic effect at the OMM. In those tumour cells, the balance of the dual activities of I κ B α is disturbed so that it exerts only its anti-apoptotic effect aided by its accumulation at the OMM as a NF- κ B target gene (Fig 2, Supplementary Fig S8A). I κ B α knock-down in such cells sensitised them for apoptosis and reduced tumour burden in a mouse model (Fig 7).

Constitutive active NF- κ B is targeted by pharmacological interference in inflammatory and autoimmune diseases (Karin *et al*, 2004). Given that the majority of solid tumours activate NF- κ B, this protein complex is also a target in cancer therapy (Baud & Karin, 2009). These efforts, however, are complicated by the immunomodulatory effects of the transcription factor. Our findings on I κ B α as a novel and major regulator of the VDAC/HKII complex that exerts an anti-apoptotic activity, which can be functionally and structurally separated from NF- κ B inhibition, reveal more specific interference options and provide a rationale for the development of targeted therapies to disrupt this complex.

Materials and Methods

Cell culture and plasmid transfection

293T, 293FT, MCF7 (Metkar *et al*, 2003), HeLa, 3T3, 3T3 I κ B α ^{−/−} (Beg *et al*, 1995), MEF WT, HCT-116 and MDA-231 cells were maintained in DMEM (Sigma, UK) and PC-3 in RPMI medium

containing 10% FCS (Sigma), 2 mM glutamine (Invitrogen, UK), 100 μ g/ml streptomycin, 100 U/ml penicillin (Invitrogen) and sodium pyruvate (Invitrogen). 293T, 3T3 and MEF cells were transfected using X-fect (Clontech, UK); MCF7, HeLa and HCT-116 and cells were transfected with Effectene (Qiagen, UK) following the manufacturer's instructions. 3T3 WT and 3T3 I κ B α ^{-/-} cells were published (Beg *et al*, 1995; Basso *et al*, 2005). MDA-MB-231, PC3 and HCT116 cells have constitutive NF- κ B activity (Nakshatri *et al*, 1997; Dejardin *et al*, 1999; Suh *et al*, 2002; Sakamoto *et al*, 2009). 3T3 EV, I κ B α - β -actin- and MEET-I κ B α -reconstituted cells were treated with H₂O₂ (250 μ M), etoposide (150 μ M), menadione (15 μ M) and ANT transfection for 24 h and with ionomycin (10 μ M) and clotrimazole (40 μ M) for 4 h.

Reagents and antibodies

Hydrogen peroxide (H₂O₂), arsenic trioxide (As₂O₃), etoposide (E1383), clotrimazole (CTZ), menadione, ionomycin, doxorubicin, docetaxel, staurosporin and proteinase K were purchased from Sigma. Monoclonal anti- β -actin, monoclonal anti-hexokinase II, horseradish peroxidase-conjugated (HRP) goat anti-rabbit and mouse monoclonal anti-hexokinase II were from Sigma-Aldrich. Antibodies for VDAC1 (N18, D16 and monoclonal), I κ B α (C-21), TOM20, GRP75, Hsp60 and GAPDH were from Santa Cruz Biotechnology (Tebu-Bio, UK). Cytochrome *c* and cyclophilin D (Ppif) and CK1 antibodies were purchased from BD Biosciences (UK). Polyclonal anti-PARP and rabbit monoclonal anti-hexokinase II were supplied from Cell Signaling (UK). HRP goat anti-mouse antibody was obtained from Jackson Laboratories (USA) and goat anti-rabbit Alexa Fluor 555 and 594 or goat anti-mouse Alexa Fluor 488 antibodies were from Molecular Probes (Invitrogen). As NF- κ B inhibitor, we used N-Oleyoldopamine (ALEXIS, Lonza, UK) (Sancho *et al*, 2004).

DNA constructs

Mammalian expression vectors coding for human Bax, human I κ B α and human RIP1 were from Origene (USA). Plasmids for mitoNEET's, Smac/DIABLO's and MCRI's mitochondria localisation sequences were obtained from Geneservice (UK), and amplified sequences were fused with I κ B α using recombinant PCR. ANT1-HA, VDAC1-HA, Caspase-8-HA, tBID-HA as well as the rest of the plasmids were cloned into pcDNA3 (Invitrogen). The Super-Repressor-I κ B α (SR-I κ B α) and I κ B α -110A3 were kind gifts from L. Schmitz, Giessen, Germany. The I κ B α -110A3 protein contains alanine substitutions at leucine 115, leucine 117 and isoleucine 120 in I κ B α . The reporter plasmid pNF κ B-hrGFP was purchased from Stratagene (UK).

Mitochondria isolation and treatment

Mitochondria were isolated with an established protocol (Gogvadze *et al*, 2003), re-suspended in the protocol's mitochondria buffer and kept at -80°C. For greater mitochondria purity but smaller yield, a commercial kit from Pierce (Thermo Fisher, UK) was also used. 25 ng/ml of proteinase K (Sigma) was applied to isolated mitochondria for 30 min at 4°C, and PMSF was used to stop the reaction. Cleavage of outer mitochondria membrane (OMM) proteins was observed on Western blots. Recombinant Bax was purchased from

ProSpec-Tany TechnoGene LTD (Israel), diluted with mitochondria buffer and incubated with isolated mitochondria for 30 min at 25°C. Transfected mitochondria with I κ B α were used or *in vitro*-translated (IVT) proteins using the TNT Quick Coupled Transcription-Translation (Promega, UK). IVT proteins were run on an SDS-PAGE for their integrity determination, and equal amounts were applied on isolated mitochondria for 30 min at 25°C prior to addition of recombinant Bax (Sukumaran *et al*, 2010). Association of IVT proteins to isolated mitochondria and the cytochrome *c* release induced by recombinant Bax protein from isolated mitochondria were analysed as described previously (Kim *et al*, 2006).

Quantification of cell death

Mitochondria dyes TMRE (tetramethylrhodamine, ethyl ester) and DiOC₆ (3,3'-dihexyloxycarbocyanine iodide) were used in conjunction with propidium iodide to quantify apoptosis (Majewski *et al*, 2004; Chiara *et al*, 2008). In short, floating and adherent cells were harvested and centrifuged at 2,100 rpm. Supernatant was discarded and cells were re-suspended in 150 μ l of PBS where DiOC₆/PI mix was added. Cells were incubated in total for 90 min and were then analysed using FACS (BD Biosciences) with the CellQuest programme (BD Biosciences). Where indicated, PI was used after permeabilisation of the cells with lysis buffer (0.1% sodium citrate, 0.1% Triton X-100 in PBS) and sub-G1/G0 DNA content was measured by FACS to quantify cell death. FlowJo (TreeStar Inc.) was used for data analysis. For qualitative analysis of apoptosis, PARP cleavage was measured.

Immunofluorescence

Cells, seeded on coverslips (CS), were transfected by mitoCFP, mitoGFP or mitoYFP with Effectene (Qiagen) and grown for 24 h, then fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. After a blocking step with 3% BSA in Tris-HCl 50 mM, NaCl 155 mM, pH 7.6 and 0.02% saponine (TBSS) for 30 min at room temperature (RT), cells were incubated with the primary antibody for 1 h at RT. Cells were rinsed five times in TBSS and then incubated with the secondary anti-rabbit Alexa Fluor 594 antibody at a dilution of 1/500 in TBSS. Cells were washed five times in TBSS and incubated 30 min in DAPI at 2 μ g/ml (Invitrogen) at RT, before being mounted in polyvinyl alcohol mounting medium with DABCO (Sigma). Cells were examined with confocal laser-scanning microscope (Leica) with a 63 $\times$ objective and analysed with LAS AF software.

Immunoprecipitation and Western blotting

Treated or untreated cells were lysed in RIPA buffer [150 mM NaCl, 50 mM Tris pH 8.0, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 2 $\times$ proteases inhibitor mixture (Sigma)]. Cell lysates were incubated on ice for 30 min and cleared by centrifugation at 4°C for 30 min at 13,000 rpm followed by two incubations of 30 min protein G magnetic beads (Millipore, UK). Cells lysates were incubated overnight at 4°C with 10 μ g of anti-I κ B α goat polyclonal Ab. Immune complexes were precipitated with magnetic protein G beads, which were washed with RIPA buffer. For VDAC1-HKII interaction experiments, VDAC1 antibodies (D-16 or N-18) were

used to immunoprecipitate complexes from isolated mitochondria following the protocol above. For FLAG immunoprecipitations, a monoclonal antibody 4C5 from Origene, Rockville, MD, USA was used. 1–2% of the total lysate was loaded for the 'Input' lanes. Immunoprecipitated proteins were separated in a 10% or 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore). Membranes were blocked with 3% BSA in TBS containing 0.1% Tween-20 and incubated with the appropriate antibodies. Specific signals were revealed by the ECL detection reagent (Pierce, UK).

Lentiviral production

Virulent media were produced in HEK293FT cells (Invitrogen) using the following optimised protocol for lentiviral production. For each 10-cm dish, plasmids encoding pVSV-G (5.4 μ g, Invitrogen), pRev (3.8 μ g, Invitrogen), pGag.Pol (7.8 μ g, Invitrogen), pAdVantage (9 μ g, Promega), and pLKO.1-shG.O.I. Vector (20 μ g) or pLenti7.3-G.O.I. vectors (20 μ g, Invitrogen) were mixed, replenished to 437.5 μ l with water, and 62.5 μ l of 2 M CaCl₂ was added. After 5 min, the DNA-calcium mix was transferred dropwise to 2 $\times$ HBS (500 μ l) and was added immediately to the cells. Following overnight incubation, the medium was replaced with DMEM containing 1 mM sodium butyrate (Sigma). The supernatant was collected 48 h post-transfection, centrifuged and filtered (0.45- μ m pore size, Millipore). 3T3 I κ B α ^{-/-} cells were transduced for 6 h in the presence of polybrene (6 μ g/ml, Sigma) for the reconstitution with I κ B α variants (pLenti7.3-G.O.I.). After infection, cells were sorted through a BD FACSARIAIIu fluorescence-activated cell sorter for eGFP since the pLenti7.3 vector allows the expression of a fluorescent marker along with the gene of interest through a bi-cistronic vector system.

I κ B α and VDAC1 downregulation by shRNA

Commercial shRNA constructs for I κ B α from Sigma were tested with Western blots for sufficient downregulation of I κ B α and two constructs were validated. The VDAC1 construct (also from Sigma) was validated with the same technique. The appropriate controls were also purchased from Sigma. The vectors were packaged into lentiviruses using the same approach as described above. Transductions of cells with constitutively active NF- κ B activity (MDA-MD-231, PC3) and non-constitutively active (HeLa) were performed with the help of polybrene, and stable cell lines were established. The sequences of the synthetic oligonucleotides (Invitrogen) used for I κ B α shRNA constructs and VDAC1 were the following:

sh-I κ B α (NFKBIA)-42 (targeting the 3' UTR):

5'-CCGGGACGAGAAAGATCATTTGAAATCTCGAGATTTCAATGATCTTTCTCGTCTTTTT-3'

sh-I κ B α -(NFKBIA)-88 (targeting the CDS):

5'-CCGGAGAGTCAGAGTTTACGGAGTTCTCGAGAACTCCGTGAAC TCTGACTCTTTTTT-3'

sh-VDAC1 (targeting the CDS):

5'-CCGGGCTTGGTCTAGGACTGGAATTCTCGAGAATTCAGTCCTA GACCAAGCTTTTT-3'

sh-VDAC1 (targeting CDS):

5'-CCGGGCAGTTGGCTACAAGACTGATCTCGAGATCAGTCTTG TAG CCAACTGCTTTTTG-3'

sh-mVDAC1 (targeting the CDS):

5'-CCGGACCAGGTATCAAAGTACGTTCTCGAGAACGTCAGTTTG AT ACCTGGTTTTTT-3'

sh-NFKBIB- I κ B β (targeting CDS):

5'-CCGGCGACTTGGAGAAGGAAGAAAGACTCGAGTCTTCTTCCTTCT CCAAGTCGTTTTT-3'

scrambled shRNA sequence:

5'-CCGGCCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACT TAACCTTAGGTTTTT-3'

Quantitative real-time PCR

To validate gene expression changes observed in cells with reduced I κ B α , we performed quantitative real-time PCR on total RNA isolated from MDA-MB-231 and PC3 cells. RNA was extracted from treated cells using the RNeasy extraction kit (Qiagen), and cDNA synthesis was performed as described. Quantitative PCR (Q-PCR) was performed on the QuantStudio™ 12K Flex Real-Time QPCR System using Taqman probes (Applied Biosystems, Life Technologies) specific to the coding regions of each of the genes assessed. Samples were run in duplicate, and GAPDH expression levels were used as an internal control for normalisation of cDNA content. The QuantStudio 12K Flex V1.1.2 software was used to analyse Q-PCR data.

Animal work

Six-week-old female Balb/c^{nu/nu} mice were injected with 2×10^6 of MDA-MB-231/_{sc} and MDA-MB-231/_{shI κ B α} cells bilaterally and in the mouse fat pad using an insulin syringe. Matrigel was used and mixed with the cells at a 1:1 ratio. Animals were anaesthetised using isoflurane. A pilot study indicated small difference in the ability of cells to grow with the MDA-MB-231/_{shI κ B α} cells growing faster 3 weeks post-inoculation. There was no difference in growth of both cell lines *in vitro*. The treatment groups were not balanced in respect to the tumour volumes as each mouse received sc and KD cells bilaterally. Three weeks post-inoculation and irrespective of tumour growth treatment with doxorubicin or PBS started by administration through the i.v. route. Mice were mock- or doxorubicin-treated once weekly, and tumour measurements were taken twice weekly using an electronic calliper. Tumour sizes were calculated using the formula $\pi/6 \times \text{length} \times \text{width} \times \text{height}$. Graphs represent average sizes of the tumour from a cohort of six mice each, and error bars show the standard error of the means. Mice with unilateral growth were excluded. Animals were treated under the 70/7113 project license following the 'Animals' (Scientific Procedures) Act 1986.

NF- κ B activity assay in 293T and 3T3 I κ B α -reconstituted cells

293T cells were transfected with the reporter plasmid pNF κ B-hrGFP along with expression constructs for ANT1, Bax and Tax (Munoz & Israel, 1995). The pan-caspase inhibitor zVAD (MPBio, UK) was applied at 20 μ M to avoid late degradation of proteins through apoptosis by Bax and ANT1. 24 h post-transfection, 293T cells were collected for FACS analysis. For the rest of the cell lines, a non-radioactive biotinylated-oligo pull-down assay was used to quantify the level of NF- κ B activity. In short, sense and antisense oligonucleotides encoding tandem κ B sites (sense strand, AGCTTA-CAAGGGACTTTCCGCTGGGGACTTTCCAGGG) were annealed after

denaturing and slow cooling in a buffered solution. The sense strand was biotinylated in the 5' position, while a non-biotinylated oligonucleotide was used as control. Nuclear extracts were prepared with standard protocols and incubated with annealed oligonucleotides for 60 min at 4°C. The oligonucleotides were subsequently precipitated by streptavidin agarose beads (Invitrogen) and rinsed three times before proceeding to Western blot analysis.

Statistical analysis

Statistical analysis was performed using the unpaired Student's *t*-test. Data were obtained from $n > 3$ experiments for every figure and were regarded as statistically significant if $P < 0.05$ (*), 0.01 (**) and 0.001 (***) based on Student's *t*-test.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

EP designed and performed experiments and analysed the data. A-LM-M, CD, WC, M-SH, JK, DQ and FO performed experiments. AA-R, AA-A, NDM, EOA and SG analysed the data. EP and SG wrote the manuscript. Stefan Grimm passed away unexpectedly after acceptance of this paper for publication. The EMBO Journal editorial team offers our condolences and sympathies to his family and coworkers on his untimely death.

Conflict of interest

The authors declare that they have no conflict of interest.

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