**Electronic Supplementary Information File**

**for**

# The natural product vioprolide A exerts anti-inflammatory actions through inhibition of its cellular target NOP14 and downregulation of importin-dependent NF-κB p65 nuclear translocation

Luisa D. Burgers[1], Betty Luong[1], Yanfen Li[3], Matthias P. Fabritius[4,5], Stylianos Michalakis[3], Christoph A. Reichel[4], Rolf Müller[6] and Robert Fürst[1,2*]

[1]Institute of Pharmaceutical Biology, Faculty of Biochemistry, Chemistry and Pharmacy, Goethe University, Frankfurt, Germany

[2]LOEWE Center for Translational Biodiversity Genomics (LOEWE-TBG), Frankfurt, Germany

[3]Department of Ophthalmology, University Hospital, Ludwig-Maximilians Universität München (LMU), Munich, Germany

[4]Department of Otorhinolaryngology and Walter Brendel Centre of Experimental Medicine, Clinical Centre of Ludwig-Maximilians Universität München (LMU), Munich, Germany

[5]Department of Radiology, University Hospital, Ludwig-Maximilians Universität München (LMU), Munich, Germany

[6]Department of Microbial Natural Products, Helmholtz-Institute for Pharmaceutical Research Saarland, Helmholtz Center for Infection Research and Department of Pharmacy at Saarland University, Saarbrücken, Germany

*Corresponding author:

Robert Fürst, Ph.D.

Institute of Pharmaceutical Biology, Goethe University Frankfurt, Max-von-Laue-Str. 9, 60438 Frankfurt, Germany

Phone: +49-69-798-29655

Fax: +49-69-798-763-29655

E-mail: fuerst@em.uni-frankfurt.de

**Fig. S1**

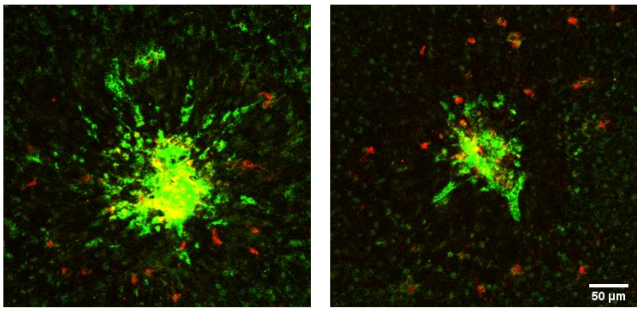


**Fig. S1** Representative merged images of CNV lesions in eye cup flat mounts prepared from vioA and vehicle-treated mice on day 14 of laser injury. Microglia/macrophage were immunostained with Iba1 antibody and CNV lesions were visualized with IB4 antibody (green). Scale bar, 50 µm.

**Fig. S2**

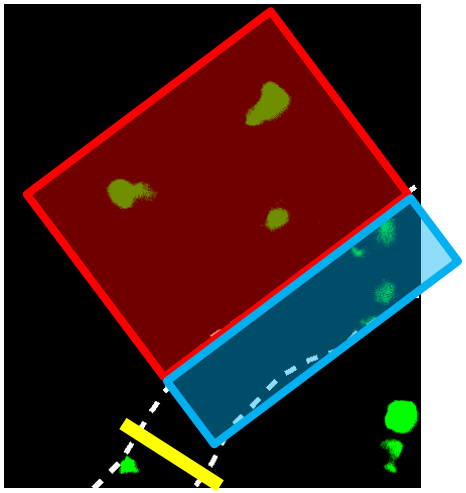

**Fig. S2** Example for quantification of rolling flux (over yellow line), adherence (in blue vessel segment along a vessel length of 100µm), and transmigration (in red perivascular area covering 100 x 75 µm) of Gr-1$^{+}$ neutrophils and classical monocytes in the mouse cremaster muscle.

**Fig. S3**

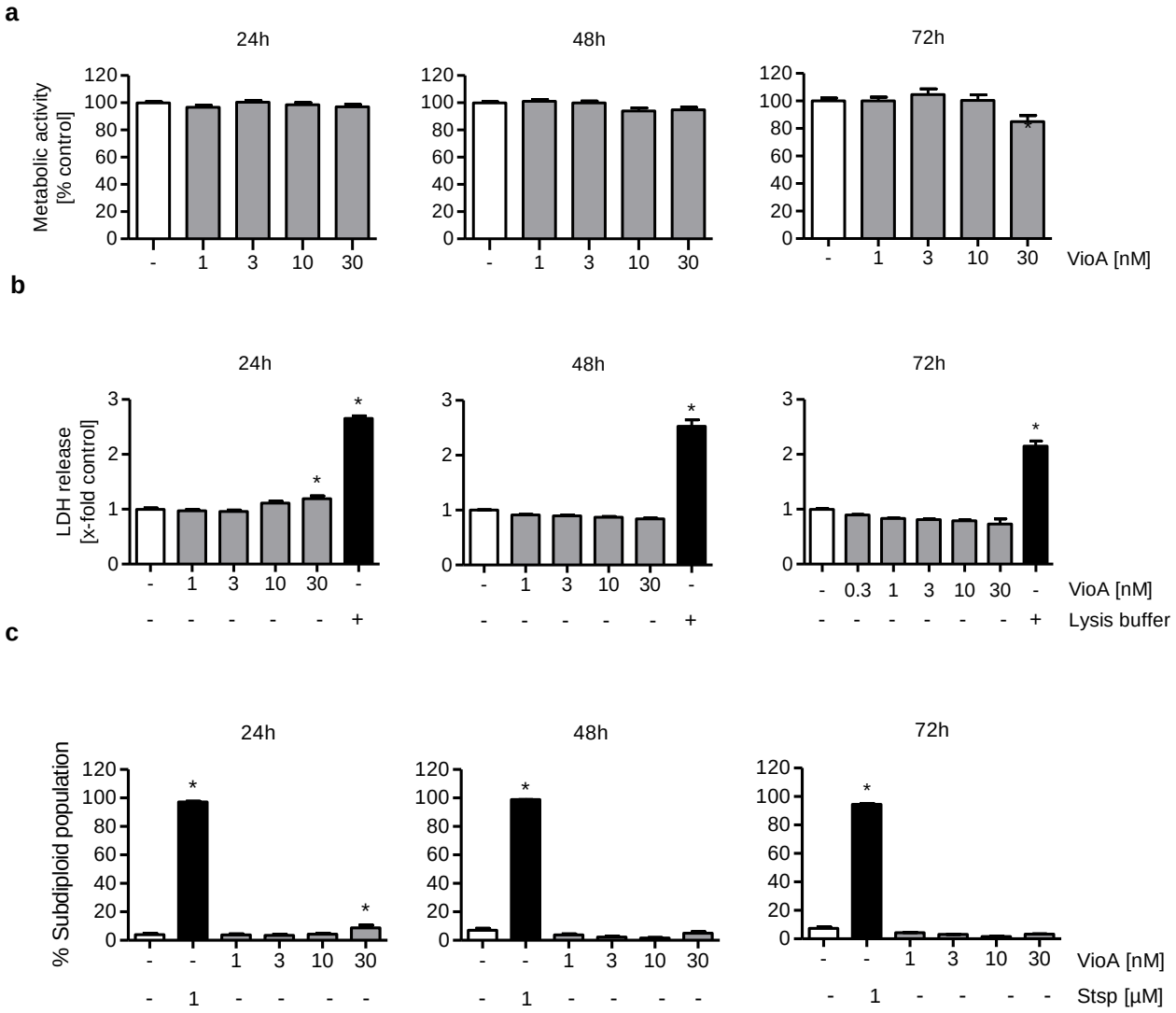


**Fig. S3** Vioprolide A does not impair cell viability of HUVECs up to concentrations of 10 nM. Confluent HUVECs were treated with vioprolide A as indicated for 24 h, 48 h or 72 h. **a** Metabolic activity was measured using the CellTiter-Blue Cell Viability Assay. Fluorescence was measured at 590 nm. **b** Lysis buffer was used as positive control to damage cell membrane integrity. Lactate dehydrogenase release was determined using the Cytotox 96 non-radioactive cytotoxicity assay. Absorbance was measured at 490 nm. **c** Staurosporine was used as positive control for inducing apoptosis. HUVECs were stained with propidium iodide. Late apoptosis was measured by determining the number of sub-diploid events via flow cytometry. VioA, vioprolide A; LDH, lactate dehydrogenase; Stsp, staurosporine. Data are expressed as mean ± SEM. n=3. *P ≤ 0.05 vs. negative control.

**Fig. S4**

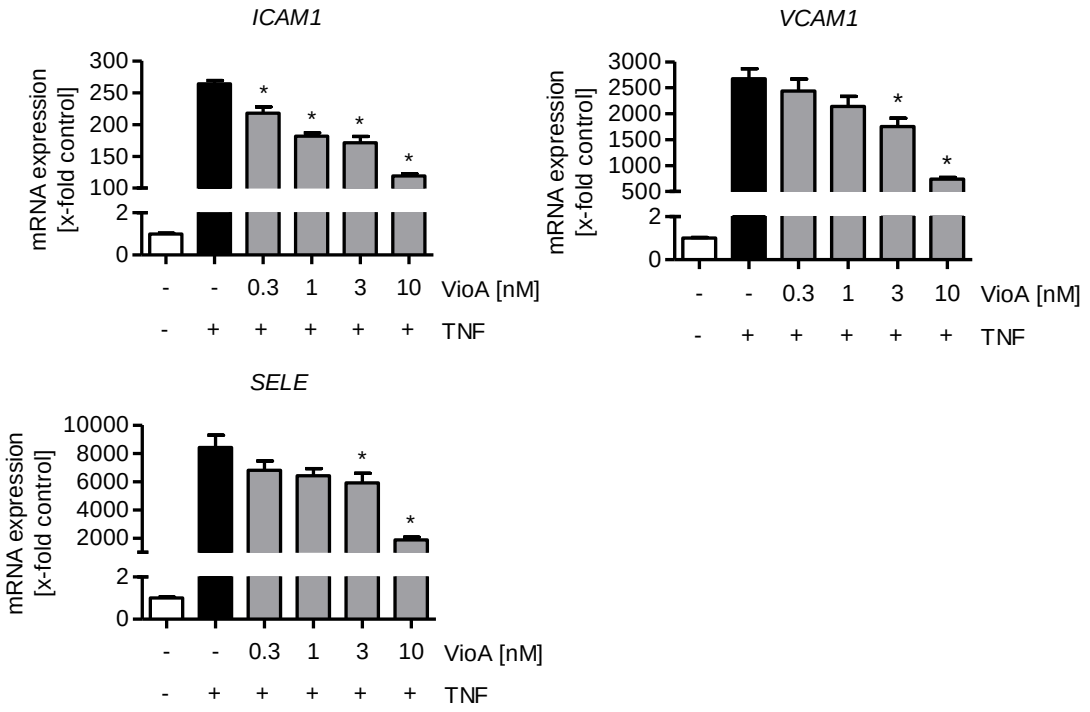


**Fig. S4** Vioprolide A impairs CAM gene expression after 4 h (*SELE*) and 6 h (*ICAM1*, *VCAM1*) of TNF treatment. Confluent HUVECs were treated as indicated for 16 h followed by TNF activation (10 ng/ml) for 4 h (*SELE*) or 6 h (*ICAM1*, *VCAM1*). The mRNA expression was analyzed by quantitative PCR. VioA, vioprolide A. Data are expressed as mean ± SEM. n=3 (*SELE*), n=4 (*ICAM1*, *VCAM1*). *P ≤ 0.05 vs. TNF control.

**Fig. S5**

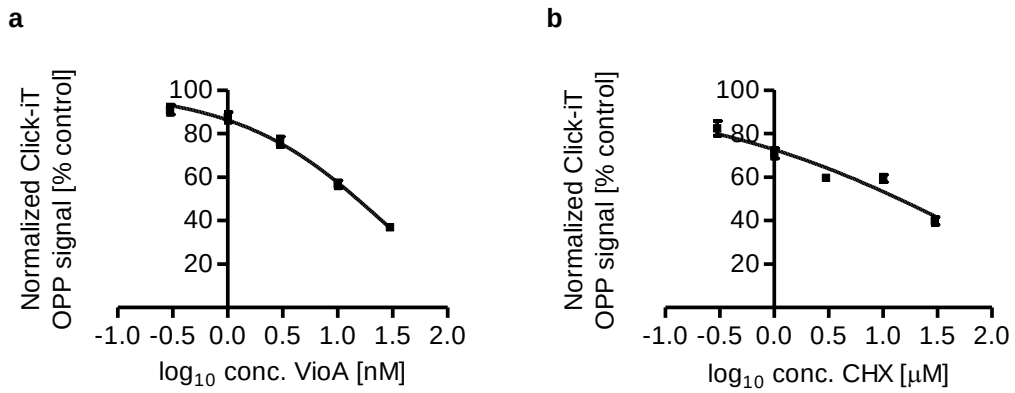


**Fig. S5** Dose response curve for the effect of vioprolide A and cycloheximide on *de novo* protein synthesis in HUVECs. Confluent HUVECs were treated with vioprolide A (**a**) or cycloheximide (**b**) for 24 h as indicated. The $IC_{50}$ was determined by analyzing nascent polypeptide chains with immunocytochemistry using Click-iT Plus OPP Alexa Fluor 488 and fluorescence microscopy. Three independent experiments were used to calculate the respective $IC_{50}$ of translation inhibition of vioprolide A and cycloheximide in HUVECs. VioA, vioprolide A; CHX, cycloheximide.

**Fig. S6**

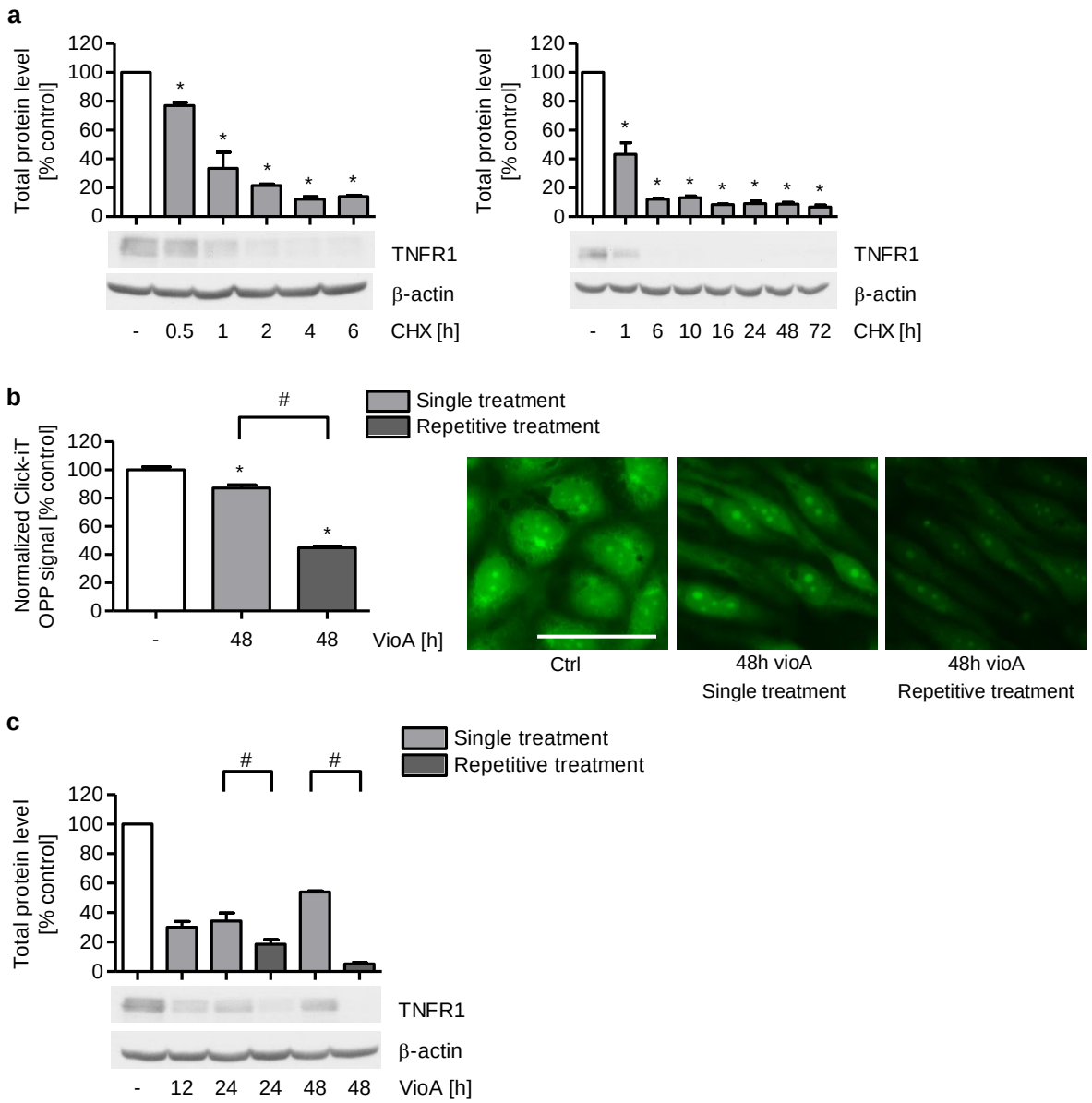


**Fig. S6** Influence of cycloheximide and vioprolide A on the TNFR1 protein level. **a-b** Confluent HUVECs were treated with cycloheximide (3 µg/ml) as indicated. Total protein expression of TNFR1 was determined by western blot analysis. One representative blot out of 3 is shown. **c** Confluent HUVECs were treated with vioprolide A (10 nM) for a total of 48 h either as single treatment of repetitive treatment each 12 h. Nascent polypeptide chains were visualized by immunocytochemistry using Click-iT Plus OPP Alexa Fluor 488 and fluorescence microscopy. One representative experiment out of 3 is shown. Scale bar, 50 µm. **d** Confluent HUVECs were treated with vioprolide A (10 nM) as indicated either as single treatment or repetitive treatment each 12 h. Total protein expression of TNFR1 was analyzed by western blot experiments. One representative blot out of 3 is shown. VioA, vioprolide A; CHX, cycloheximide. Data are expressed as means ± SEM. n=3. *P ≤ 0.05 vs. negative control; #P ≤ 0.05 vs. single treatment.

**Fig. S7**

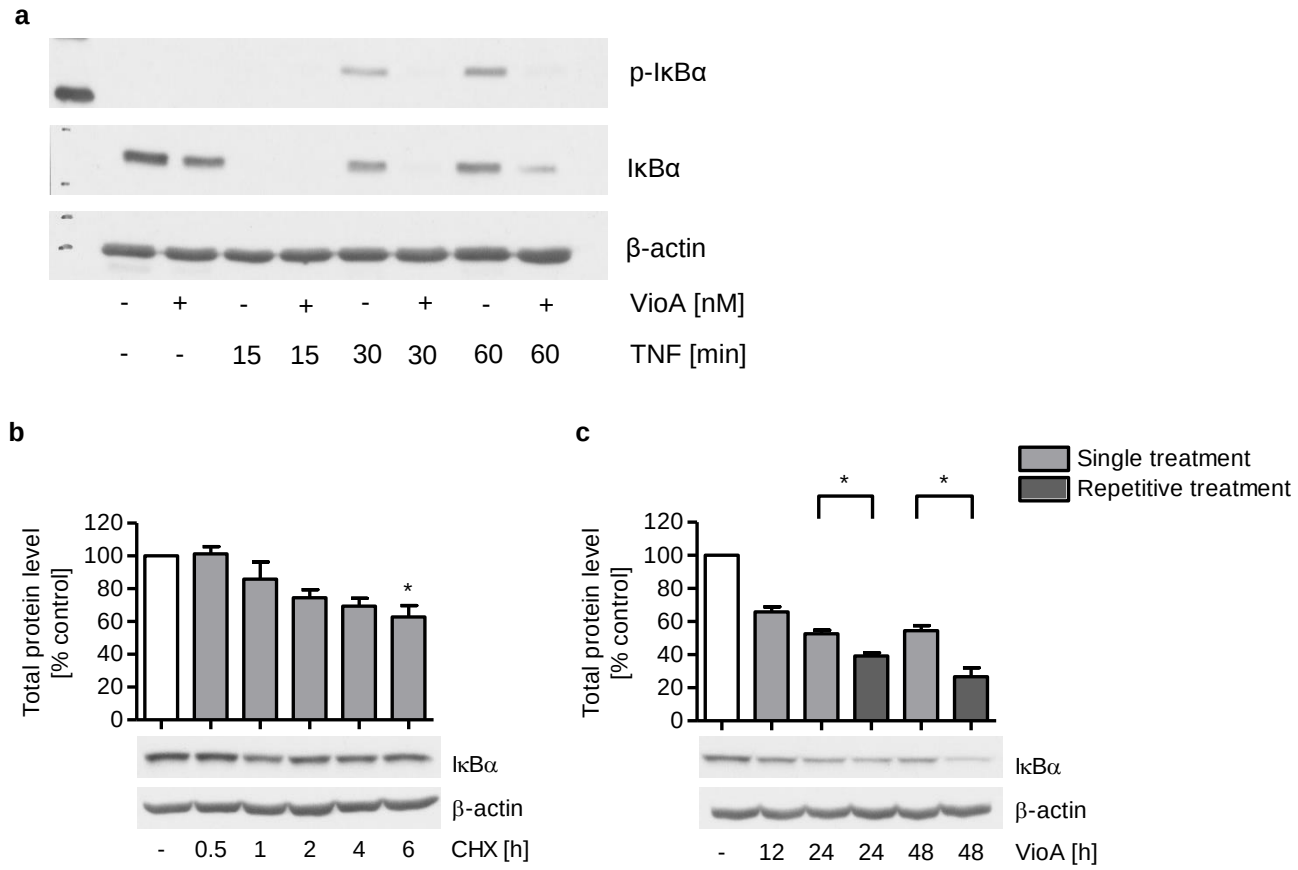


**Fig. S7** Influence of vioprolide A on the TNF-induced phosphorylation and TNF-independent protein level of IκBα in HUVECs. **a** Confluent HUVECs were treated with vioprolide A (10 nM) for 16 h followed by activation with TNF (10 ng/ml) for the indicated time points. Total protein expression of phospho-IκBα (p-IκBα) was analyzed by western blot experiments. One representative blot out of 3 is shown. **b** Confluent HUVECs were treated with cycloheximide (3 µg/ml) as indicated. Total protein level of IκBα was determined by western blot analysis. One representative blot out of 3 is shown. **c** Confluent HUVECs were treated with vioprolide A (10 nM) as indicated either as single treatment or repetitive treatment each 12 h. Total protein level of IκBα was determined by western blot analysis. One representative blot out of 3 is shown. VioA, vioprolide A; CHX, cycloheximide. Data are expressed as mean ± SEM. n=3. *P ≤ 0.05 vs. negative control (**b**) or single treatment (**c**).

**Fig. S8**

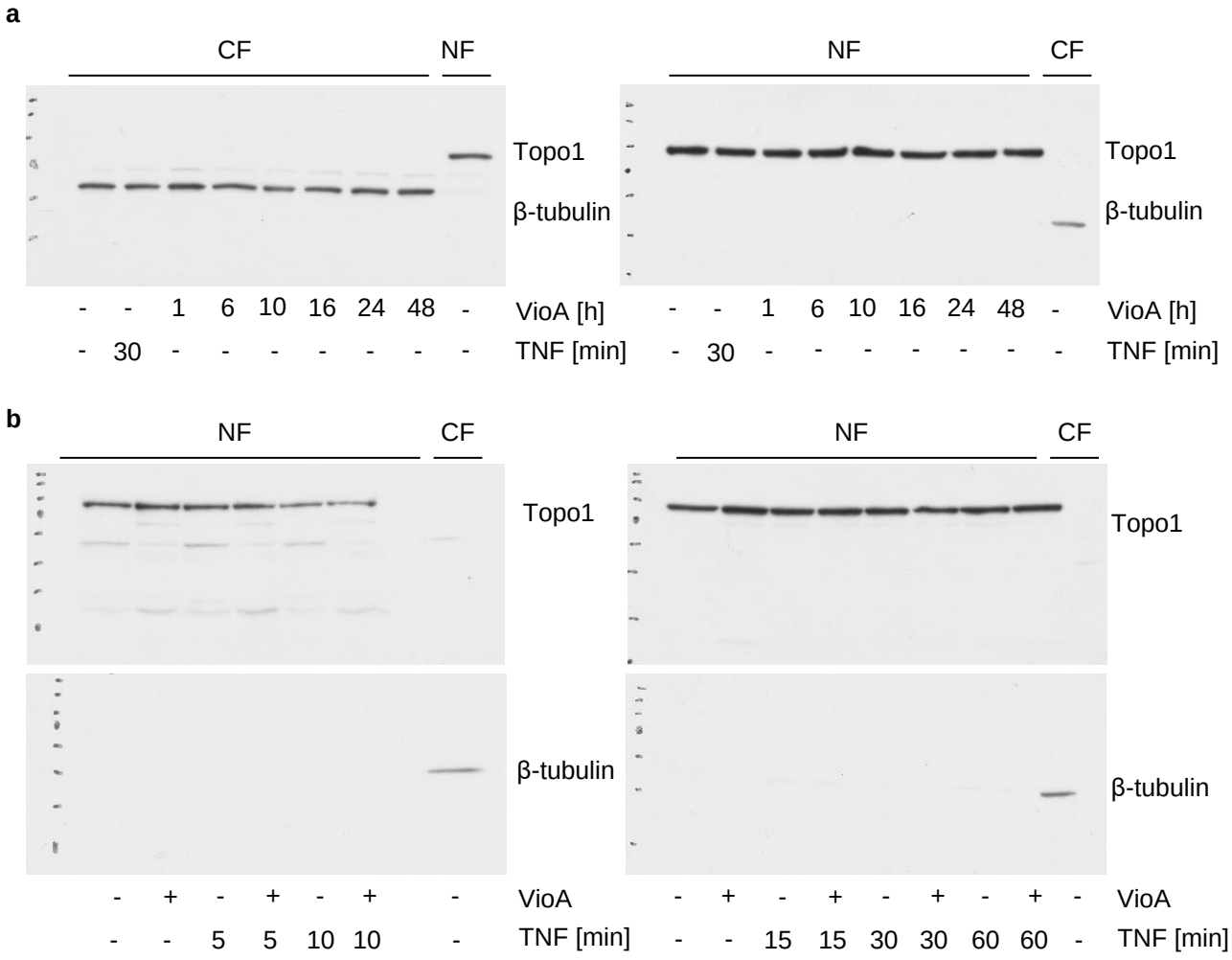


**Fig. S8** Purity of the cytosolic and nuclear fraction after cell fractionation. **a** Confluent HUVECs were treated with vioprolide A (10 nM) or TNF (10 ng/ml) as indicated. Cytosolic and nuclear fractions were separated. Purity of the subcellular fractions was verified using western blot analysis. One representative blot out of 3 is shown. **b** Confluent HUVECs were treated with vioprolide A (10 nM) for 16 h followed by activation with TNF (10 ng/ml) as indicated. The nuclear fraction was separated and its purity was verified using western blot analysis. One representative blot out of 3 is shown. CF, cytosolic fraction; NF, nuclear fraction; VioA, vioprolide A; Topo1, topoisomerase 1. n=3.

**Fig. S9**

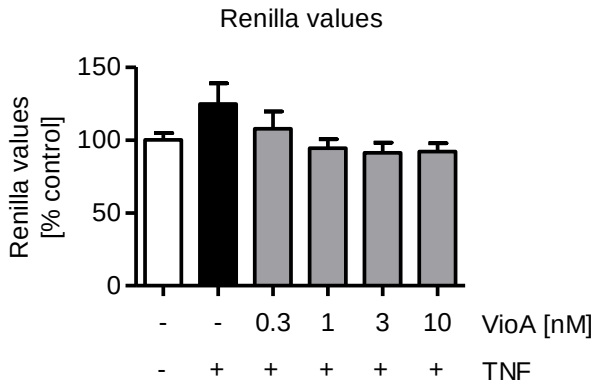


**Fig. S9** Vioprolide A has no influence on the expression of *Renilla* luciferase. HUVECs were co-transfected with a vector coding for the NF-κB response element and firefly luciferase and a control vector coding for *Renilla* luciferase. 24 h post transfection, HUVECs were treated with vioprolide A as indicated for 16 h followed by activation with TNF (10 ng/ml) for 6 h. *Renilla* luciferase activity was measured by dual-luciferase reporter gene assay and luminescence measurement. Data are expressed as mean ± SEM. n=4.

**Fig. S10**

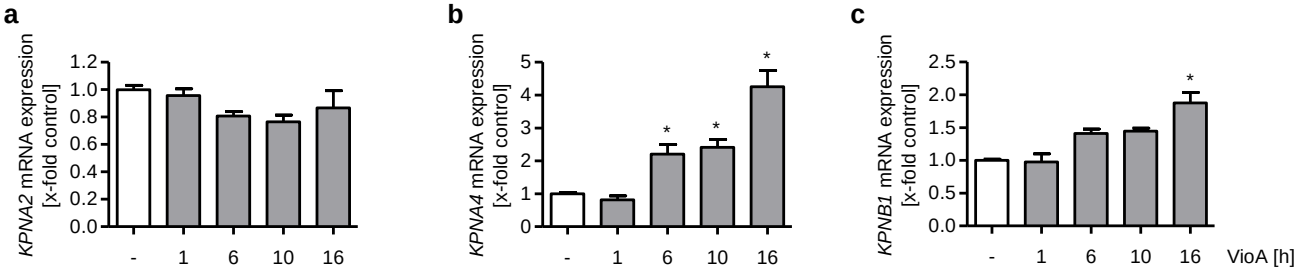


**Fig. S10** Vioprolide A has no influence on KPNA2 mRNA expression but increases *KPNA4* and *KPNB1* mRNA levels. Confluent HUVECs were treated with 10 nM vioprolide A as indicated. Total mRNA expression of *KPNA2* (**a**), *KPNA4* (**b**) and *KPNB1* (**c**) was measured with quantitative PCR. vioA, vioprolide A. Data are expressed as mean ± SEM. n=3. *P ≤ 0.05 vs. TNF control.

**Fig. S11**

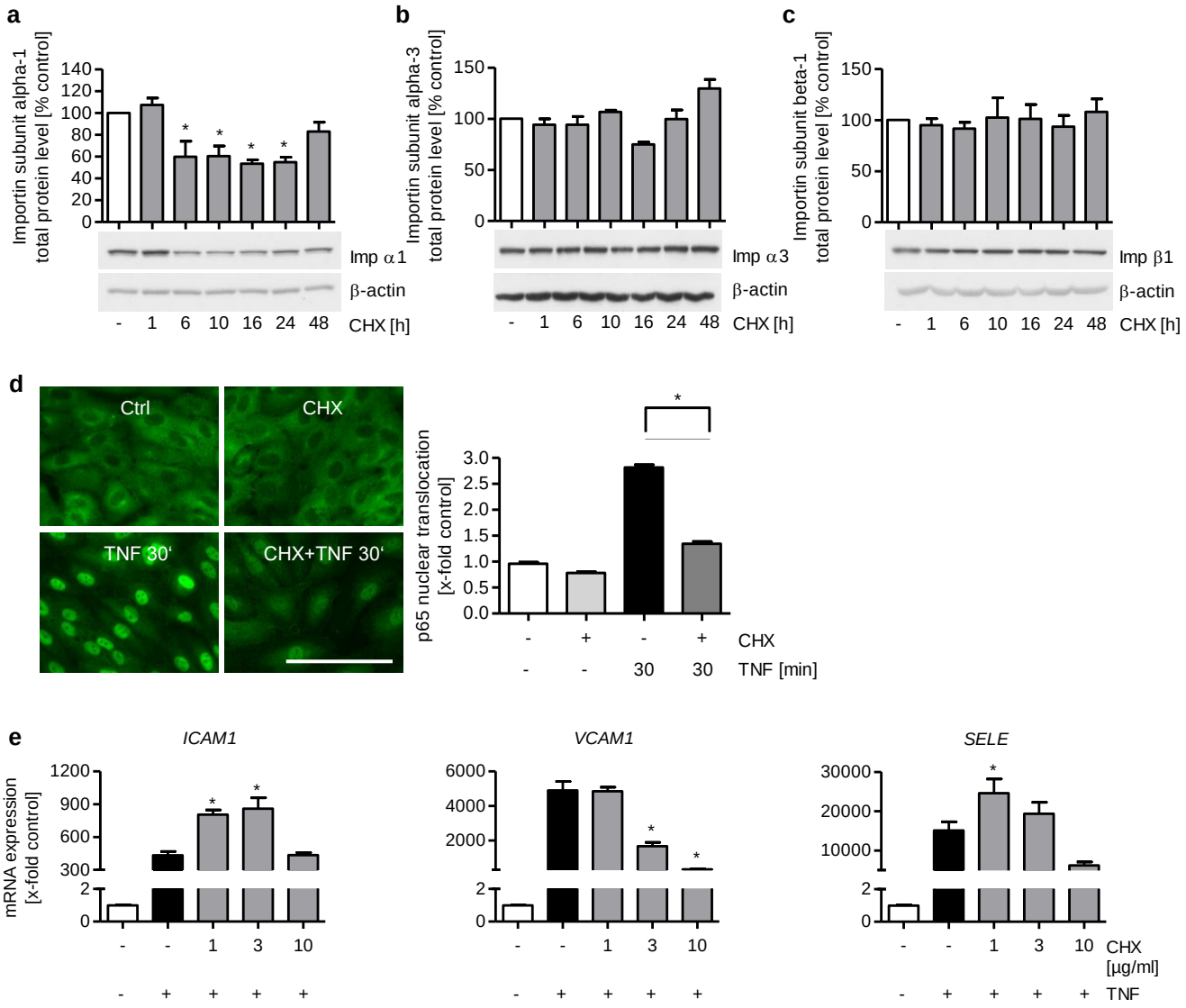


**Fig. S11** Effects of cycloheximide on the total protein level of importins, p65 nuclear translocation and target gene expression. **a-c** Confluent HUVECs were treated with 3 µg/ml cycloheximide as indicated. Total protein levels of importin subunit alpha-1 (**a**), importin subunit alpha-3 (**b**) and importin subunit beta-1 (**c**) were determined by western blot. One representative blot is shown. **d** Confluent HUVECs were treated with 3 µg/ml cycloheximide for 16 h followed by activation with 10 ng/ml TNF for 30 min. p65 was visualized by immunocytochemistry and fluorescence microscopy. One representative experiment out of 3 is shown. Scale bar, 200 µm. **e** Confluent HUVECs were treated for 16 h with cycloheximide as indicated followed by activation with TNF (10 ng/ml) for 2 h (*SELE*) or 4 h (*ICAM1*, *VCAM1*). The mRNA expression of *ICAM1*, *VCAM1* and *SELE* was analyzed by quantitative PCR. CHX, cycloheximide; Imp, importin. Data are expressed as mean ± SEM. n=3. *P ≤ 0.05 vs. control (**a-c**) or TNF control (**d-e**).

**Fig. S12**

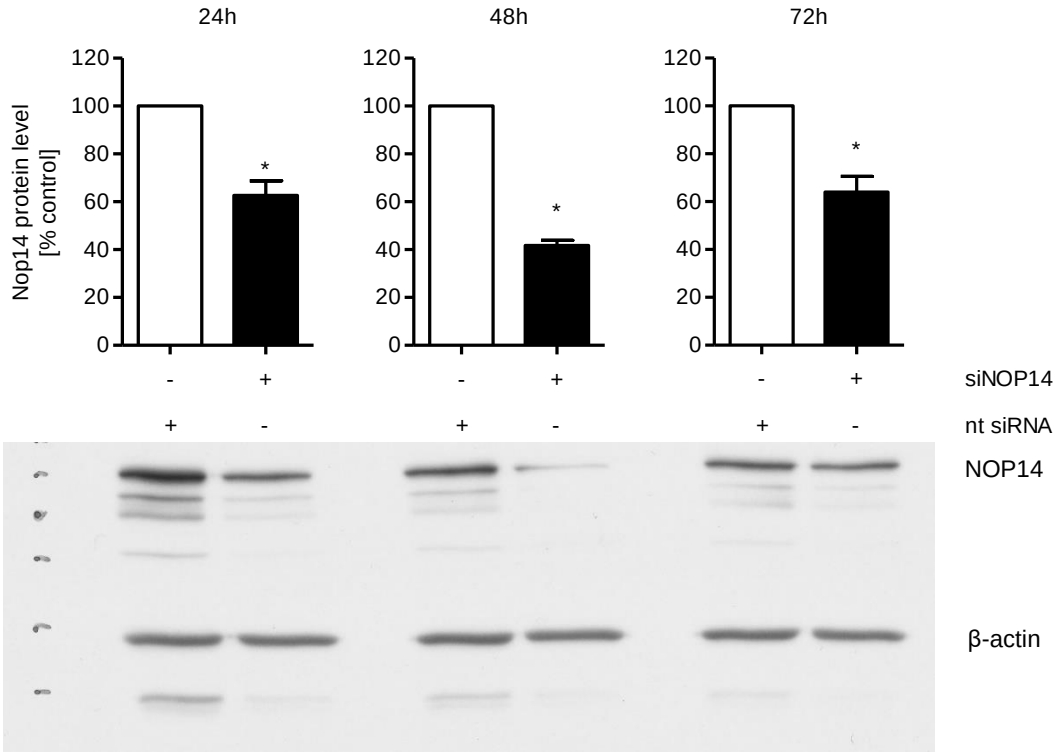


**Fig. S12** Knockdown of NOP14 in HUVECs. Subconfluent cells were transfected with siRNA against NOP14 (siNOP14, 60 nM) or non-targeting siRNA (nt siRNA, 60 nM) using GeneTrans II Transfection Reagent. 24 h, 48 h and 72 h after transfection, total protein expression of NOP14 was analyzed using western blot experiments. One representative blot out of 3 is shown. Data are expressed as mean ± SEM. n=3. *P ≤ 0.05 vs. negative control.