1   **A NOVEL MECHANISM FOR NF-κB-ACTIVATION VIA IκB-AGGREGATION: IMPLICATIONS**

2   **FOR HEPATIC MALLORY-DENK-BODY INDUCED INFLAMMATION**

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4   **Yi Liu[1], Michael J. Trnka[2], Shenheng Guan[2], Doyoung Kwon[1], Do-Hyung Kim[3], J-J. Chen[4],**

5   **Peter A. Greer[5], A. L. Burlingame[2], and Maria Almira Correia[1,2,6,7]**

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7   Departments of Cellular & Molecular Pharmacology[1], Pharmaceutical Chemistry[2], and

8   Bioengineering and Therapeutic Sciences[6], and The Liver Center[7],

9   University of California San Francisco, San Francisco CA 94158-2517,

10   Departments of Biochemistry, Molecular Biology, and Biophysics[3], University of Minnesota,

11   Minneapolis, MN55455,

12   Institute for Medical Engineering and Science [4], MIT, Cambridge, MA, 02139

13   Department of Pathology and Molecular Medicine[5], *Queen's* University; Kingston, ON; K7L 3N6

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15   **Running title:** *NF-κB-activation upon PPIX-elicited IκB-sequestration*

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17   Address correspondence to: M. A. Correia, Mission Bay Campus, Genentech Hall, 600 16th
18   Street, Box 2280, University of California San Francisco, San Francisco, CA 94158-2517.
19   FAX: 415-476-5292. E-mail: almira.correia@ucsf.edu

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23

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30
31   **Author Contributions**:

32   Y.L and M.A.C designed the studies and wrote the manuscript. M.A.C supervised the project.

33   Y.L conducted most of the experiments with MS support and interpretation from M.T. and S.G.

34   D.K. carried out the ZnPP *in vivo* mouse experiments. D.Y.K, J.J.C, and P.A.G provided critical,

35   *sine qua non* research materials. All authors critically reviewed the manuscript.

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47 **ABSTRACT**

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49 **Background & Aims:**

50 Mallory-Denk-bodies (MDBs) are hepatic protein aggregates associated with inflammation both

51 clinically and in MDB-inducing models. Similar protein aggregation in neurodegenerative

52 diseases also triggers inflammation and NF-κB activation. However, the precise mechanism that

53 links protein aggregation to NFκB-activation and inflammatory response remains unclear.

54

55 **Methods:**

56 Herein, we find that treating primary hepatocytes with MDB-inducing agents (N-

57 methylprotoporphyrin, protoporphyrin IX (PPIX), or ZnPPIX) elicited an IκBα-loss with

58 consequent NF-κB activation. We characterized the underlying mechanism in detail using

59 hepatocytes from various knockout mice and MEF cell lines and multiple approaches including

60 immunoblotting, EMSA, RT-PCR, confocal immunofluorescence microscopy, affinity

61 immunoprecipitation, and protein solubility assays. Additionally, we performed rigorous

62 proteomic analyses to identify the proteins aggregating upon PPIX treatment and/or co-

63 aggregating with IκBα.

64

65 **Results:**

66 Four known mechanisms of IκBα-loss were probed and excluded. Immunofluorescence

67 analyses of ZnPPIX-treated cells coupled with 8 M urea/CHAPS-extraction revealed that this

68 IκBα-loss was due to its sequestration along with IκBβ into insoluble aggregates. Through

69 proteomic analyses we identified 47 aggregation-prone proteins that co-aggregate with

70 IκBα through direct interaction or proximity. Of these ZnPPIX-aggregation targets, the

71   nucleoporins Nup153 and Nup358/RanBP2 were identified through RNA-interference, as likely

72   mediators of IκBα-nuclear import.

73

Conclusion:

75   We discovered a novel mechanism of inflammatory NF-κB activation through IκB-sequestration

76   into insoluble aggregates along with interacting aggregation-prone proteins. This mechanism

77   may account for the protein aggregate-induced inflammation observed in MDB-associated liver

78   diseases, thereby identifying novel targets for therapeutic intervention. Because of inherent

79   commonalities this MDB cell model is a *bona fide* protoporphyric model, making these findings

80   equally relevant to the liver inflammation associated with clinical protoporphyria.

81

Lay Summary:

83   Mallory-Denk-bodies (MDBs) are hepatic protein aggregates commonly featured in many liver

84   diseases. MDB-presence is associated with the induction of inflammatory responses both

85   clinically and in all MDB-inducing models. Similar protein aggregation in neurodegenerative

86   diseases is also known to trigger inflammation and NFκB pathway activation via an as yet to be

87   characterized non-canonical mechanism. Herein using a MDB-inducing cell model, we

88   uncovered a novel mechanism for NFκB activation via cytosolic IκB-sequestration into insoluble

89   aggregates. Furthermore, using a proteomic approach, we identified 47 aggregation-prone

90   proteins that interact and co-aggregate with IκBα. This novel mechanism may account for the

91   protein aggregate-induced inflammation observed in liver diseases, thereby identifying novel

92   targets for therapeutic intervention.

93

94 **INTRODUCTION**

95   Protein aggregates and inclusion bodies are linked to various neurodegenerative,

96   muscular and hepatic diseases [Alzheimer's, Parkinson's, Desmin-related myopathies, and

97   Mallory-Denk-bodies (MDBs) [1]]. Although different aggregates vary with each tissue source

98   and in predominant protein composition, they contain common components such as

99   p62/Sequestosome-1, ubiquitin, chaperones and proteasome constituents, which are often

100   misfolded, highly insoluble, and cross-linked [1, 2].

101   Hepatic MDBs consisting largely of phosphorylated cytokeratins and p62 are commonly

102   found in patients with alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH),

103   primary biliary cirrhosis, non-alcoholic cirrhosis, hepatocellular carcinoma, morbid obesity, and

104   copper-related disorders [3]. MDBs can be reproduced in mice by long term feeding of

105   griseofulvin (GF) or 3,5-dicarbethoxy-1,4-dihydrocollidine (DDC) [4, 5]. Both compounds

106   inactivate certain hepatic cytochromes P450, converting their prosthetic heme into N-

107   methylprotoporphyrin(s) (NMPP) that inhibit hepatic ferrochelatase (Fech) resulting in hepatic

108   heme depletion and protoporphyrin IX (PPIX) accumulation [6, 7] (Fig. 1A). $Fech^{m1Pas}$ mutant

109   (*fech/fech*) mice with <5% of normal enzyme activity also develop spontaneous MDBs at 20-

110   weeks of age, validating their use as an experimental model for MDB induction [8].

111   ASH and NASH patient liver histology reveals that MDBs are associated with

112   inflammatory responses, with MDB-containing hepatocytes often surrounded by neutrophils [1,

113   9]. All MDB-inducing mouse models also accumulate hepatic PPIX and exhibit liver inflammation

114   and injury at an early stage when MDBs are not yet visible [10, 11]. Cumulative biochemical

115   evidence indicates that in these mouse models as well as in DDC-, NMPP- or PPIX-treated cell

116   models, certain proteins such as p62, cytokeratins CK8/18 and lamin begin to aggregate at a

117   much earlier stage before MDBs are detected [12, 13]. Similarly, many neurodegenerative

118   diseases are also associated with inflammation at an early stage, and amyloid protein

119 aggregation has been shown to initiate an inflammatory response [14]. Moreover, induction of

120 various protein aggregates, as in myofibrillar myopathy and Amyotrophic Lateral Sclerosis (ALS),

121 triggers the activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells)

122 via a noncanonical pathway independent of IκBα-phosphorylation/degradation [15]. However,

123 the precise molecular link between protein aggregation and NF-κB-activation and inflammatory

124 response remains unclear.

125 Of the existing NF-κB/Rel transcription factors [16], p65/p50 is not only the most

126 ubiquitous and biologically active NF-κB-heterodimer, but also the major hepatic species [17].

127 p65/p50 is normally sequestered in the cytoplasm by NF-kappa-B inhibitors (IκB). Of these, in

128 human hepatocytes, HepG2 cells and cultured mouse hepatocytes, IκBα and IκBβ are the major

129 forms, each regulated by different signals [18-21]. IκB-binding masks NF-κB nuclear localization

130 signal (NLS) and DNA-binding domain [16]. Signal-induced IκB-unleashing of cytoplasmic NF-

131 κB most commonly via phosphorylation and subsequent ubiquitin-dependent degradation (UPD)

132 of both IκBα and IκBβ [22-24], results in the nuclear translocation of DNA-binding competent

133 NF-κB, with consequent transcriptional activation of its target genes including that of IκBα [25],

134 but not of IκBβ [26].. IκBα is then rapidly *de novo* oversynthesized (overshoot), and following

135 nuclear import, binds and displaces the DNA-bound NF-κB, masking its NLS and accelerating

136 its nuclear export, required for down regulation and eventual termination of NF-κB-mediated

137 transcriptional activation [27]. Thus, while both hepatic IκBs are largely involved in NF-κB-

138 cytoplasmic retention, only IκBα is involved in nuclear NF-κB-transcriptional suppression [19]

139 Our findings herein reveal a loss of IκBα and IκBβ with subsequent NF-κB activation

140 upon treatment of primary hepatocytes with NMPP (an established MDB inducer), or ZnPPIX

141 (ZnPP). We probed the role of four plausible mechanisms for NF-κB-activation via reduction of

142 intracellular IκBα-levels in this NMPP-elicited IκBα-loss and excluded them all (Supplementary

6

143 Results). Instead, we found that this apparent IκBα-loss upon NMPP- or ZnPP-treatment is

144 actually due to its phase sequestration into insoluble cellular aggregates along with IκBβ, as well

145 as the nucleoporins Nup153 and Nup358 that are involved in IκBα nuclear import and

146 subsequent termination of NF-κB activation.

147 To our knowledge, this is the first evidence that NF-κB is also activated through IκB-

148 sequestration into insoluble aggregates. We believe such a novel mechanism accounts for the

149 persistent hepatic NF-κB activation that may directly contribute to the severe inflammatory

150 responses and liver injury observed not only in various MDB-featuring liver diseases and

151 experimental MDB-models but also in acute erythropoietic protoporphyria (EPP) and X-linked

152 protoporphyria (XLPP) [28-30].

153

154 **RESULTS**

155 ***NMPP-elicited PPIX accumulation with concurrent IκBα-loss and NF-κB activation in***

156 ***cultured mouse hepatocytes:*** NMPP-treatment of cultured mouse hepatocytes as expected

157 from its ferrochelatase inhibition, resulted in cellular PPIX accumulation (Fig. 1A). In parallel, a

158 gradual IκBα loss was also observed in hepatic lysates upon NMPP-treatment (Fig. 1B). This

159 IκBα-loss was reversed upon inclusion of hemin, but not that of the proteasomal inhibitor MG-

160 132, or the dual autophagic-lysosomal degradation (ALD) inhibitors 3-methyladenine (3MA) +

161 NH$_4$Cl. These findings thus excluded both UPD and ALD involvement in this IκBα-loss. Further

162 supportive evidence was provided by studies in HepG2 cells transfected with a S$_{32}$A/S$_{36}$A-IκBα

163 mutant that is resistant to phosphorylation and UPD (Fig. S1A) as NMPP caused a similar time-

164 dependent loss of both WT IκBα and its mutant. Parallel EMSA of nuclear extracts from these

165 NMPP-treated hepatocytes revealed a time-dependent hepatic NF-κB activation, indicating that

166 this hepatic IκBα loss was indeed physiologically relevant (Figs. 1C & S1B). This NF-κB

167 activation was also attenuated by hemin (Fig. 1C), consistent with the hemin-mediated inhibition

168 of IκBα loss (Fig. 1B). These findings suggested that this culture model was a valid model to

169 interrogate the association of PPIX accumulation with NF-κB-activation and inflammation.

170

171 Four additional mechanisms -- two established and two plausible -- for NF-κB-activation via

172 reduction of intracellular IκBα-levels [i.e. (i) translational suppression by heme-deficiency-

173 triggered heme-regulated inhibitor (HRI) eIF2α kinase-activation ([31]; Figs. 1D & S2A); (ii)

174 enhanced autophagy ([32]; Fig. S2B); (iii) enhanced calpain-mediated proteolysis ([33];

175 Fig.S2C); and (iv) PPIX-photoactivation and consequent ROS-mediated oxidative stress [28] in

176 NMPP-elicited IκBα-loss (Supplementary Results; Fig. S3), were all probed and excluded.

177

178 ***PPIX and ZnPP are even more potent inducers of IκBα-loss, NF-κB-activation and p62***

179 ***oligomerization/aggregation than NMPP:*** Because NMPP-elicited ferrochelatase inhibition

180 results in PPIX-accumulation, we examined whether this accumulation (and not heme

181 deficiency) was mainly responsible for the observed IκBα loss and NF-κB activation. We

182 observed a greater IκBα loss after either PPIX or ZnPP relative to that seen with NMPP alone

183 (Fig. 1E). While the NMPP-elicited IκBα loss was to a great extent prevented by hemin

184 inclusion, the IκBα loss after PPIX or ZnPP was not (Fig. 1E; see Supplementary Discussion).

185 EMSA analyses revealed a NF-κB activation that was proportional to the observed IκBα loss,

186 ranking in order as follows: ZnPP>PPIX>NMPP (Fig. 1F). Consistent with this NF-κB activation,

187 RT-PCR analyses of mRNA from ZnPP-treated hepatocytes indicated marked up-regulation of

188 the inflammatory cytokine IL-6 at 24 h (Fig.1G).

189   Because p62 is a major component of MDB and various other pathological aggregates

190 [1], we monitored hepatic p62 response upon NMPP, PPIX and ZnPP ± hemin treatments (Figs.

191 1B, 1E). In parallel with IκBα loss, p62 formed aggregates and possibly cross-linked species,

192 with a concurrent decline in the monomeric species (~62 kD; Ctrl), evident at later (8 h) rather

193 than earlier (2-4 h) time points. This apparent time-lag is most likely due to an initial

194 counteractive compensatory p62 transcriptional induction in response to PPIX-elicited oxidative

195 stress and Nrf2 activation [8]. Furthermore, we found that the p62-monomer levels correlated

196 reasonably well ($r^2 = 0.72$) with corresponding IκBα levels in these treated and untreated cells,

197 revealing an intimate association between IκBα loss and p62-aggregation, possibly due to their

198 direct protein interactions (Fig. S1D).

199

200 ***ZnPP-elicited IκBα-loss is independent of p62 or other autophagic receptors/adapters:***

201 The capacity of p62 to stabilize IκBα upon co-expression in HEK293T cells (Fig. 2A) and the

202 close temporal relationship between IκBα-loss and p62 aggregation (Fig. 1B) suggested these

203 proteins were intimately associated. We thus examined their interdependence in ZnPP-treated

204 p62 WT and p62 null mouse (p62KO) hepatocytes or MEF cells (Figs. 2B & S4A), and found

205 that this IκBα loss was independent of p62 aggregation. The additional possibility that in p62-

206 lacking cells, IκBα loss was due to its degradation via UPD, ALD or calpain pathways was

207 excluded with specific inhibitors of these pathways (Fig. S4B). We also examined plausible

208 compensation by NBR1 (Neighbour of Braca 1 gene) [34] (Fig S4C, S4D), and three other

209 autophagic receptors/adapters [35], and conclusively excluded p62 and its redundant functional

210 mimics in this ZnPP-elicited IκBα-loss (Fig. 2D).

211

212 ***Cytoplasmic IκBα-loss is due to its physical sequestration into insoluble cellular***

213 ***aggregates:*** Confocal microscopic immunofluorescence (CMIF) analyses of ZnPP-treated WT

214 and p62$^{-/-}$-MEF cells provided an informative clue (Fig. 2E). In WT-cells, both proteins were

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215   largely colocalized to the cytoplasm, and ZnPP did not affect this colocalization (Fig. 2E, insets).

216   Surprisingly, following ZnPP-treatment, in spite of immunochemical IκBα-loss (Fig. 2D), IκBα-

217   associated immunofluorescence signal still persisted comparably in both WT and p62$^{-/-}$-MEF

218   cells. This finding provided the first clue that upon ZnPP-treatment, IκBα was not irretrievably

219   lost, but just undetectable in detergent-solubilized cell lysates routinely employed for

220   immunoblotting (IB) analyses. It was thus likely that such an IκBα physical inaccessibility was

221   due to intracellular ZnPP-triggered protein aggregation. To examine this possibility, HEK293T

222   cells co-transfected with both HA-IκBα and p62-Myc plasmids, were treated with ZnPP and

223   then sequentially extracted using detergents of increasing strengths (Supplementary Methods).

224   IB analyses showed that upon ZnPP-treatment, in parallel with p62 aggregation, HA-

225   IκBα became undetectable in soluble Triton and RIPA fractions (Fig. 3A), however, the majority

226   of HA-IκBα as well as monomeric p62 and p62-aggregates could be recovered from the RIPA-

227   insoluble pellet by heat extraction with 8M urea/CHAPS buffer (urea buffer; Supplementary

228   Methods). Furthermore, similar results were found upon IB analyses of these urea extracts

229   irrespective of whether antibodies to the HA-tag, N- or C-terminus of IκBα were employed (Fig.

230   3B). IB analyses of high salt buffer (HSB)-extracts from ZnPP-treated cell lysates also revealed

231   detectable monomeric and aggregated IκBα and p62 species, albeit to a much lesser extent

232   than corresponding urea extracts (Fig. 3C). Together, these findings indicated that upon ZnPP-

233   treatment, p62 and IκBα along with other cellular proteins co-aggregate, and these aggregates

234   are sequestered from the cytoplasm into an insoluble cellular fraction, essentially unavailable to

235   function physiologically.

236

237   ***Proteomic identification of plausible IκBα-interactors and ZnPP-aggregated proteins:***

238   PPIX-treatment is causally associated with the highly selective aggregation of many cellular

239   proteins [36]. Thus, IκBα could either be the prime target of ZnPP-elicited physical sequestration

240   or a silent partner dragged along for the ride by one or more of its cellular interactors. To identify

241   any plausible mediators of ZnPP-elicited IκBα sequestration into insoluble cellular aggregates,

242   we employed a proteomic approach to comprehensively identify the proteins that normally

243   interact with IκBα, but which form protein co-aggregates upon ZnPP-treatment. These

244   aggregation-prone proteins upon IκBα-interaction could drive its sequestration and co-

245   aggregation.

246   To obtain a high-confidence IκBα-interactome, we performed rigorous immunoaffinity

247   purification coupled with mass spectrometry (IAP-MS) analyses, with extensive biological

248   repetitions and strict filtering (Fig. 4A, 4B, Supplementary Methods). This approach identified

249   370 high-confidence IκBα-interactors, including well-established IκBα-interactors [i.e. NF-κB

250   subunits (RelA, NFKB1, NFKB2), and IKK-complex proteins (CHUK, IKBKAP)] (Fig. 4C), thus

251   validating our IAP-MS approach. However, most of the proteins identified were novel IκBα-

252   interactors.

253   Previous studies in *fech/fech* mouse and protoporphyric zebra fish models have

254   identified several (≈ 30) aggregated proteins in the high molecular mass (HMM)-regions upon

255   SDS-PAGE of NP40-soluble and/or SDS-soluble liver fractions [8, 13, 36, 37]. Our discovery

256   that ZnPP-elicited protein aggregates could be resolubilized by heating in urea buffer, prompted

257   comprehensive proteomic analyses of these urea-solubilized protein aggregates. For this

258   purpose, the progressive temporal course of this protein aggregation process was followed in

259   HEK293T and HepG2 cells treated with ZnPP for 2 h and 24 h (Fig. 4D). The entire urea-

260   solubilized fraction as well as the HMM-bands from SDS-PAGE of Triton and RIPA soluble

261   extracts were subjected to LC-MS/MS proteomic analyses (Fig. 4D, E). Overlap analyses

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262   revealed that 192 proteins commonly aggregated in both cell lines upon ZnPP-treatment (Fig.

263   4F).

264

265   ***Disruption of multiple functional networks upon ZnPP-elicited physical sequestration of***

266   ***IκBα and other cellular proteins:*** To gain functional insight into these 192 ZnPP-aggregated

267   proteins and their possible contribution to cell toxicity, we undertook a comprehensive protein

268   network analyses using the Search Tool for the Retrieval of Interacting Genes/Proteins

269   (STRING) coupled with biological process and pathway overrepresentation analyses (Fig. 5A).

270   Clearly, ZnPP-elicited physical aggregation clustered these proteins under 4 critical biological

271   processes. Those proteins occupying a network hub/node position within their individual clusters,

272   could disrupt multiple vital cellular interactions, leading to global hepatic functional collapse.

273   Additional overlap of the 370 IκBα-interacting proteome with the common 192 ZnPP-aggregate

274   proteome identified 47 IκBα-interacting proteins in common with the ZnPP-aggregate proteome

275   (*each highlighted in yellow*; Fig. 5A). By contrast, well-established IκBα interactors (RelA,

276   NFKB1, NFKB2, CHUK and IKBKAP) were not detected within the ZnPP-aggregate proteome of

277   either cell type (Fig. 5A). An enrichment ratio of the 47 overlapping proteins under each

278   experimental condition was then employed to percentile rank them in a heat-map clustering

279   format (Fig. 5B). Such analyses identified many nuclear pore complex (NPC) nucleoporins

280   Nup153, Nup155, and Nup93, and Nup358/SUMO E3-ligase RANBP2, as significantly enriched

281   in this combined proteome (Fig. 5B).

282   To further verify that these 47 proteins were indeed co-aggregating with IκBα, rather

283   than merely aggregating in parallel, we employed biotinylation proximity labeling to identify

284   IκBα-interacting proteins within the protein aggregates. After due consideration of various

285   proximity labeling approaches, we selected HRP-biotinylation by antibody recognition (BAR) [38]

286   for identification of IκBα-interacting proteins in both soluble and insoluble fractions of ZnPP-

287   treated and untreated cells (Fig. S5). The overlapping proteome identified via both co-IP and

288   BAR approaches revealed a very high-confidence IκBα-interactome. More importantly, of the

289   previously identified 47 IκBα-interacting proteins in common with the ZnPP-aggregate proteome,

290   37 were also detected via BAR to be interacting with IκBα in ZnPP-triggered protein aggregates

291   (Fig 5B), indicating that they were indeed co-aggregating.

292

293   ***Nucleoporin Nup153 is a principal IκBα-interactant and ZnPP-target:*** The heat-map

294   clustering analyses of these 47 proteins consistently identified the nuclear pore basket

295   component Nup153 as the top common IκBα-interactant in IAP-MS, BAR, and HEK293 and

296   HepG2 ZnPP-aggregate proteome. We therefore verified Nup153 presence through IB analyses

297   of Triton- and urea-solubilized cellular aggregates upon a 2 h-ZnPP-treatment of HEK293 and

298   HepG2 cells.   Indeed, whereas native Nup153 exhibited its intrinsic 153 kDa-mobility in

299   untreated cells, upon ZnPP-treatment it was found as HMM-protein aggregates in Triton- and

300   urea extracts, along with endogenous IκBα (Fig. 6A). Furthermore, co-immunoprecipitation (Co-

301   IP) using HEK293T cells revealed a significant fraction of endogenous Nup153 interacted with

302   IκBα both in cytoplasmic and nuclear extracts of GFP-IκBα-transfected cells, but not in mock- or

303   C1-GFP-transfected cells (Fig. 6B). A similar interaction was also evident among the

304   endogenous counterparts under basal conditions. However, this Nup153-IκBα-interaction was

305   greatly enhanced upon TNFα-treatment at times (1 and 1.5 h) when rapid translocation of *de*

306   *novo* synthesized IκBα across the nuclear pore would be expected to trigger its post-induction

307   repression of NF-κB-activation (Fig. 6C). Furthermore, ZnPP-treatment of TNFα-pretreated cells

308   led to accelerated IκBα sequestration, whose timing revealed that ZnPP may preferentially

309   target *de novo* synthesized IκBα as it increasingly interacts with cytoplasmic Nup153 during its

13

310   nuclear import (Fig. 6C-D). Consistent with this, our CMIF of HepG2 cells revealed that under

311   basal conditions, both NF-κB (p65-Rel A subunit) and IκBα are localized in the cytoplasm (Fig.

312   6E). But upon TNFα-treatment, IκBα UPD results in NF-κB nuclear translocation within 0.5 h.

313   Subsequently, upon transcriptional activation of the NF-κB-responsive IκBα-gene, newly

314   synthesized IκBα enters the nucleus resulting in NF-κB dissociation and nuclear export, all

315   within 1 h of TNFα-treatment. By contrast, upon ZnPP-treatment, in spite of this robust IκBα-

316   restoration at 1 h after TNFα-treatment, it is apparently functionally defective as a nuclear NF-

317   κB-repressor, as NF-κB persisted in the nucleus (Fig. 6E). This stalled IκBα appears

318   prominently both in the cytoplasm as well as clustered around the outer nuclear envelope rim

319   (Fig. 6E). Additional support for this likelihood is provided by our Nup153 and RanBP2 siRNA-

320   knockdown analyses that revealed the marked attenuation of nuclear IκBα-import upon TNFα-

321   activation, without affecting its ZnPP-sequestration (Figs. 7 & S6).

322

323   ***IκBα siRNA knockdown analyses reveal that ZnPP-triggered NF-κB activation may***

324   ***additionally involve IκBβ-sequestration:*** Because of the functional redundancy of various

325   IκBs in NF-κB cytoplasmic retention, IκBα-deficiency through genetic ablation, siRNA

326   knockdown, or cycloheximide-inhibition of IκBα-synthesis fails to increase constitutive NF-κB

327   transcriptional activation in many nonhepatic cell-types [19, 39, 40]. Thus, upon our IκBα

328   siRNA-knockdown, a similar slight, barely detectable constitutive NF-κB activation was

329   observed in HepG2 cells (Fig. 8A, left panel), whereas in primary mouse hepatocytes this

330   activation was quite substantial (Fig. 8A, right panel). However, it was not quite as marked as

331   that observed upon ZnPP-treatment, possibly due to the significant compensatory IκBβ-

332   upregulation upon IκBα siRNA-knockdown (Fig. 8B). Because in hepatocytes both IκBα and

333   IκBβ are the predominantly expressed IκBs [18, 21], and given the relatively more pronounced,

334   sustained and consistently reproducible ZnPP-elicited NF-κB activation, we determined whether

335   ZnPP also similarly sequestered IκBβ. Indeed, upon IκBα siRNA of HepG2 cells, the

336   compensatorily increased IκBβ was similarly targeted to ZnPP-elicited aggregation, resulting in

337   no appreciable mitigation of the ZnPP-induced NF-κB activation post IκBα-knockdown (Fig 8C).

338   Furthermore, immunoblotting re-analyses of the RIPA-soluble ZnPP-treated lysates revealed

339   that IκBβ also disappeared from its usual monomeric position, but was found in HMM protein

340   aggregates (Fig. 8D). Such an apparent "IκBβ-loss" was also resistant to various UPD, calpain

341   and ALD inhibitors, consistent with a sequestration process similar to that of IκBα. The ZnPP-

342   targeting of both hepatic IκBs thus accounts for its potent NF-κB activation. Accordingly, *in vivo*

343   ZnPP-treatment of mice revealed marked mRNA increases of several hepatic proinflammatory

344   NF-κB target genes i.e. IL-6, IL-1β, TNFα and osteopontin (Fig. 8D)

345

346   **DISCUSSION**

347   ***Why is IκBα vulnerable to ZnPP-elicited physical sequestration from the cytoplasm?***

348   Recent evidence increasingly supports a sequestration and co-aggregation model of

349   pathogenesis [41]. In a snowballing effect, aggregation-prone disease proteins "hijack" their

350   interacting partners with vital functions, sequestering them into cytotoxic insoluble inclusions

351   [41]. The protein scaffold p62 is one such IκBα-co-aggregating protein. However, scrutiny of

352   ZnPP-treated p62^{-/-} hepatocytes and MEF cells reveals that it is not essential for ZnPP-elicited

353   IκBα-sequestration. This is consistent with the report that in p62^{-/-} mouse liver, p62 is not

354   required for MDB formation, just for their maturation and stability [42]. These aggregation-prone

355   proteins share some common physicochemical properties: Preexistent proteins are relatively

356   large in size, enriched in domains with high intrinsic disorder or unstructured regions, and exhibit

357   low average hydrophobicity [43]; newly synthesized proteins on the other hand, are more

358   vulnerable due to prolonged exposure of their relatively hydrophobic domains either during or

359   soon after synthesis during their subsequent folding, assembly, or transport [43].

360   None of the 47 proteins that interacted with IκBα and co-aggregated upon ZnPP-

361   treatment is a well-established stable IκBα interactor. Although most of the known stable IκBα

362   interactors were repeatedly found in our co-IP analyses (Fig 4C), they were never detected in

363   the ZnPP-aggregate proteome. This suggests that these 47 proteins may be transient

364   IκBα interactors that are difficult to capture without highly sensitive and selective approaches.

365   Our analyses of the physicochemical properties of these 47 proteins indicated that they ranged

366   widely in size, with ~90% exhibiting relatively low hydrophobicity, and ~80% predicted to contain

367   a long (>30 residues) disordered segment (Table S1). These dual features of low hydrophobicity

368   coupled with high intrinsic disorder could synergistically contribute to their ZnPP-elicited co-

369   aggregation upon transient interaction, and may explain why ZnPP initially targets preexistent

370   IκBα species exhibiting both features (Table S1). The structurally related IκBβ is similarly

371   vulnerable to ZnPP-elicited sequestration thus accounting for the remarkably profound NF-κB-

372   activation. Whether this vulnerability stems from their common structural ankyrin-repeat domain

373   feature remains to be determined. Free IκBα species is apparently unstable and requires NF-κB

374   binding for folding to a stable conformation [44]. After TNFα stimulation, newly synthesized

375   IκBα would be unstable and thus greatly susceptible to ZnPP-elicited aggregation without

376   nuclear import in order to bind NF-κB and properly fold, thus resulting in the sustained ZnPP-

377   triggered NF-κB-activation.

378   Although NF-κB and IκBα form a stable complex, they are not static cytoplasmic

379   residents but shuttle continuously between the nucleus and cytoplasm [45]. Interestingly, p65

380   and IκBα translocate into the nucleus via different pathways. p65 enters the nucleus via the

381   classical NLS-and importin (α3/α4)-dependent machinery [46], whereas IκBα and several other

16

382   ankyrin repeat domain-containing proteins (ARPs) enter the nucleus via a NLS- and importin-

383   *independent* machinery [47]. This machinery depends on a direct interaction between the ARPs,

384   RanGDP as well as mobile nucleoporins such as Nup153 and RanBP2 for entry across the NPC

385   into the nucleus. Indeed, plausibly relevant to this IκBα-nucleocytoplasmic shuttling process, our

386   proteomic analyses of the 47 common IκBα-interacting and ZnPP-co-aggregating proteins

387   underscored significant enrichment of mobile NPC nucleoporins. Of these, our heat-map

388   analyses singled out Nup153 as both the top-ranked IκBα-interactant and ZnPP-aggregation

389   target (Fig. 5B). By contrast, importins α3 and α4 relevant to p65 nuclear import were not found

390   in the ZnPP-aggregate proteome, which may explain why p65 is not co-aggregated during its

391   nuclear import.

392

393   ***Potential role of Nup153 in IκBα-mediated NF-κB-repression:*** The   nucleoporin   Nup153

394   primarily exists N-terminally anchored to the nuclear pore basket while its disordered and

395   flexible FG-rich C-terminus extends into the cytoplasm [48]. Another Nup153 pool apparently

396   exists that shuttles between the cytoplasmic and NPC faces [49], and interacts directly with

397   cargos (i.e. Stat1, Smad2, and PU.1) for their nuclear import via a transporter-*independent*

398   machinery [48]. Nup153, in contrast to other FG-rich nucleoporins i.e. Nup98, Nup62, Nup214,

399   also strongly binds ARs of iASPP and ASPP2, two representative substrates of the NLS-

400   independent ARP-RanGDP nuclear import pathway [47]. Structural/biochemical analyses

401   indicate that Nup153 binds RanGDP at a higher affinity than RanGTP [50]. This collective

402   evidence suggests a critical role of Nup153 in facilitating ARP-RanGDP nuclear import. Our

403   findings that IκBα co-immunoprecipitated not only with Nup153 in the nuclear extracts (NER),

404   but also more appreciably with that in the cytoplasmic extracts (CER), even though the basal

405   cytoplasmic Nup153-content is much lower than its nuclear content (compare inputs, Fig. 6B),

17

406   are consistent with a minor, albeit highly dynamic, cytoplasmic Nup153 pool. This pool directly

407   interacts with IκBα and may be responsible for the continuous IκBα-nucleocytoplasmic traffic at

408   steady state, as indeed verified by our Nup153 knockdown analyses (Fig. 7). The concurrent

409   ZnPP-elicited aggregation of Nup153 and IκBα suggests that the basal nuclear import of

410   IκBα as well as that of newly synthesized IκBα after TNFα-stimulation is disrupted, leading to

411   NF-κB nuclear persistence and prolonged response.

412   Another noteworthy proteomic finding is our quite prominent detection of the SUMO E3-

413   ligase RanBP2 in this 47-protein cohort. RanBP2, an outer NPC component, fans its FG-rich

414   filaments into the cytoplasm [51]. The proteomic detection of RanBP2, but no other cytoplasmic

415   NPC nucleoporin i.e. Nup214 in this common 47-protein cohort is functionally intriguing because

416   besides Nup153, RanBP2 is also involved in receptor-independent nuclear import through direct

417   interactions with its specific cytoplasmic cargos [51]. This is intriguing given that IκBα

418   SUMOylation is reportedly involved in its nuclear entry and subsequent post-induction NF-κB-

419   repression [52]. Because RanBP2 also strongly binds RanGDP, conceivably a RanBP2-

420   RanGDP-Nup153-IκBα-SUMO complex is involved in IκBα-nuclear import, a possibility

421   consistent with our RanBP2 knockdown analyses (Fig. 7). The inviability of actively dividing

422   HEK293T cells precluded our simultaneous knockdown of both nucleoporins to mimic their

423   concurrent ZnPP-elicited sequestration. The failure of Nup153 or RanBP2 knockdown to affect

424   ZnPP-elicited IκBα-sequestration suggests that the newly synthesized IκBα is itself vulnerable,

425   particularly in the absence of both Nup153 and RanBP2 to chaperone its nuclear entry.

426   We find it quite instructive that many of these nucleocytoplasmic trafficking proteins as

427   well as others within the 47 co-aggregated proteome are well-established components of

428   pathogenic neurodegenerative disease protein aggregates (MS and ALS) or inclusions

429   (Parkinson's Lewy bodies), as well as hepatic MDBs (liver disease and hepatic protoporphyria

18

430   models) [Fig. 5B; [13, 36, 37, 43, 53, 54]]. For example, Nup153 was shown not only to co-

431   aggregate with cargo proteins in oligodendrocyte precursor cells in MS lesions [55], but also

432   detected as anterior horn cell cytoplasmic inclusions of an ADAR2-deficient ALS mouse model

433   [56]. These findings not only underscore the common intrinsic physicochemical and

434   physiological properties of these aggregation-prone proteins, but also suggest that their

435   interaction with IκBα could also similarly drive their co-aggregation under disease stresses, and

436   thus account for the associated tissue inflammation and injury commonly seen in both liver and

437   neurodegenerative diseases.

438

439   ***Plausible clinical relevance to EPP and XLPP:*** Such ZnPP-elicited persistent NF-κB

440   transcriptional activation may also account for the inflammation and injury stemming from

441   chronic PPIX exposure not only in Fech^{m1Pas}, DDC- and GF-protoporphyric rodent livers, but

442   also in EPP and XLPP patient livers [10, 11, 28-30, 57], wherein avid Fe-overutilization may

443   drive ZnPP-generation. ZnPP generation is also a prominent feature of iron-deficiency anemias

444   and lead poisoning (Lamola and Yamane, 1974), Although our studies were largely confined to

445   primary hepatocytes, in intact protoporphyric human and mouse livers these parenchymal cells

446   are juxtaposed to non-parenchymal Kupffer cells and hepatic stellate cells that are quite capable

447   of both canonical and non-canonical NF-κB-signaling. Thus, upon chronic PPIX-exposure, their

448   NF-κB-activation could induce proinflammatory cytokines, chemokines, growth factors and other

449   mitogens [17]. This additional paracrine cytokine stimulus could further potentiate the PPIX-

450   elicited NF-κB-signaling activation within the hepatocyte, further aggravating the extent of EPP

451   and XLPP liver injury. Indeed, exogenous TNFα greatly accelerated ZnPP-elicited IκBα-

452   sequestration in hepatocytes (Fig. 6D)

453   The precise mechanism of PPIX-induced protein aggregation remains to be elucidated.

454   Porphyrins trigger protein cross-linking through a secondary reaction between the

455   photooxidative products of histidine, tyrosine and/or tryptophan and free NH_2-groups of amino

456   acids [58]. Given the light-sheltered *in vivo* environment of the liver, such a photooxidative

457   protein crosslinking seems unlikely in PPIX-mediated hepatic MDB formation, although PPIX

458   can trigger protein aggregation and cell toxicity in the dark, albeit at a slower rate [36].

459   Additionally, porphyrins also induce structural changes in certain proteins, leading to their

460   functional impairment [59]. Such structural changes could expose some hydrophobic regions

461   thereby inducing protein aggregation. In this context, the 192 PPIX-targeted aggregate proteins

462   identified herein, we propose, could provide valuable insight into the future elucidation of the

463   precise mechanism of PPIX-induced protein aggregation.

464

465   **Experimental Procedures**

466   **Cell culture and transfections**

467   Primary mouse hepatocytes were isolated from C57BL/6 wild type male mice by collagenase

468   perfusion. HepG2, HeLa, HEK293T and MEF cells were cultured using standard methods.

469   HEK293T cells were transfected using TurboFect (ThermoFisher) and HepG2 cells were

470   transfected using X-tremeGENE HP (Roche) according to the manufacturers' instructions. See

471   Extended Experimental Procedures for details.

472

473   **EMSA**

474   Nuclear fractions of cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction

475   Reagents (ThermoFisher). EMSA were performed using LightShift Chemiluminescent EMSA Kit

476   (ThermoFisher). See Extended Experimental Procedures for details.

477

478 **Western Immunoblotting (IB) Analyses:**

479 Whole-cell extracts were prepared with Cell Lysis buffer (Cell Signaling Technology)

480 supplemented with 10% glycerol and protease/phosphatase inhibitor cocktail (Pierce). Cell

481 lysates were sonicated for 10s and then cleared by centrifugation at 4°C in a tabletop centrifuge

482 at the highest speed for 10 min. Protein concentrations were determined by BCA assay and

483 equal amounts of proteins were separated on Tris-Glycine eXtended (TGX) polyacrylamide gels.

484 Proteins were transferred onto nitrocellulose membranes (Biorad, Hercules, CA) for IB analyses.

485 See Extended Experimental Procedures for details.

486

487 **Mass spectrometry (MS):**

488 Samples subject to MS proteomics analyses were processed using standard in-gel digestion

489 and the resulting peptide mixture was desalted using C18-Ziptips (Millipore) and then injected

490 into an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher). Raw data were processed

491 using ProteinProspector (version 5.19.1; http://prospector.ucsf.edu/prospector/mshome.htm).

492 See Extended Experimental Procedures for details.

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654

655

656

657   **FIGURE LEGENDS:**

658   ***FIGURE 1. NMPP treatment results in concurrent PPIX-accumulation, NF-κB activation***

659   ***and IκBα-loss.*** **A**. A scheme for NMPP-mediated inhibition of ferrochelatase with consequent

660   accumulation of the heme precursor PPIX**.** The PPIX content of lysates from mouse

661   hepatocytes treated with two different commercial lots of NMPP for 24 h quantified

662   flurometrically (Mean ± SD, n = 3). **B**. IB analyses of p62 and IκBα with histone H3 as the

663   loading control in lysates from mouse hepatocytes treated as indicated. NMPP (30 μM), hemin

664   (10 μM added fresh every 4 h), and ALIs, autophagy/lysosomal inhibitors 3MA (5 mM) + NH_4Cl

665   (30 mM), and MG132 (20 μM) for 1 h. **C**. Mouse hepatocytes were treated as indicated.

666   Representative EMSA of nuclear extracts (NER, 5 μg) with NF-κB-specific oligonucleotides is

667   shown. Bar chart represents mean ± SD of NF-κB activation relative to controls, n = 3. **D**.

668   Quantification of IκBα levels upon IB analyses of lysates from wild type (WT) and HRI^{-/-} mouse

669   hepatocytes treated with 30 μM NMPP for the indicated times (Mean ± SD, n = 3). **E**. IB

670   analyses of p62 and IκBα in lysates from mouse hepatocytes treated with 10 μM PPIX, 10 μM

671   ZnPP or 30 μM NMPP with or without 10 μM hemin for 8 h. NS, non-specific band. **F**. EMSA of

672   nuclear extracts (NER) from mouse hepatocytes treated as indicated for 24 h. **G**. IL-6 RT-PCR

673   analyses of mRNA from mouse hepatocytes treated with 10 μM ZnPP for the indicated times

674   (Mean ± SD, n = 3).

675

676   ***FIGURE 2. ZnPP-elicited IκBα-loss is p62-independent.*** **A**. HEK293T cells were transfected

677   with pCMV4-3HA-IκBα, or co-transfected with either pcDNA6-p62-Myc or pcDNA6-Myc empty

678   vector for 48 h. Cell lysates were used for IB analyses with actin as the loading control. **B**.

679   Primary hepatocytes from wild type (p62WT) and p62 knockout (p62KO) mice were treated with

680   10 μM ZnPP for the indicated times. Cell lysates were used for IB analyses. **C**. Wild-type Hela

681   cells (WT) and CRISPR-engineered Hela cells with five autophagic adapters genetically deleted

682   (5KO) were treated with 10 μM ZnPP for the indicated times. Lysates were used for IB analyses.

683   **D**. CMIF analyses. p62WT and p62KO MEF cells were treated with 10 μM ZnPP or vehicle

684   control for 2 h, and then fixed and stained with anti-IκBα (green), anti-p62 (magenta) and DAPI

685   (Blue). Insets depict enlarged regions of IκBα and p62 colocalization.

686

687   *FIGURE 3. ZnPP-elicited IκBα-loss is due to its sequestration into insoluble cellular*

688   *aggregates.* **A**. HEK293T cells were transfected with pCMV4-3HA-IκBα for 40 h, and then

689   treated with 10 μM ZnPP for 4 h. Cells were then subjected to sequential extraction. 10 μg of

690   extracts were used for IB analyses. **B**. The same extracts from (**A**) were immunoblotted using

691   three different antibodies each targeting a different region of the same HA-IκBα protein: Anti-

692   HA-tag, rabbit monoclonal antibody targeting IκBα N-terminus (N), and rabbit monoclonal

693   antibody targeting IκBα C-terminus (C). **C**. HEK293T cells were transfected and treated as in **A**.

694   and then subjected to sequential extraction with the following buffers: Cell Signaling lysis buffer

695   (Triton), Cell Signaling lysis buffer supplemented with 1.5 M KCl (HSB). The pellet obtained after

696   HSB-extract was either solubilized with heating in a Laemmli buffer with 4% SDS (SDS) or in

697   urea buffer. 10 μg of extracts were used for IB analysis.

698

699   *FIGURE 4. IAP-MS of IκBα-interactome and ZnPP-induced aggregate proteome.* **A**. Flow-

700   chart of the strategy used to identify a high-confidence IκBα-interactome. Cells expressing GFP

701   (mEmerald) alone were used as the background control. **B**. Representative Coomassie Blue

26

702   staining lysates before and after IAP. **C**. 370 high-confidence IκBα-interactors were plotted with

703   the mean log of enrichment factor against GFP-control on the Y-axis and the *p*-value on the X-

704   axis. Previously known IκBα-interactors are labeled in red. **D**. Flow-chart of the strategy

705   employed to identify ZnPP-induced aggregate proteome of both HepG2 and HEK293T cells. **E**.

706   Coomassie Blue staining of proteins from sequential Triton, RIPA and urea fractions. HMM

707   fractions of Triton and RIPA extracts and the entire urea fraction (demarcated by the red boxes)

708   were excised from the gel for in-gel digestion and MS analyses (LC-MS/MS). **F**. Venn diagram

709   of proteins enriched at least 2-fold in ZnPP-induced aggregates relative to corresponding

710   controls from HEK293T (HEK) and HepG2 cells indicated 192 proteins as common hits.

711

712   ***FIGURE 5. STRING network analyses of proteins common to ZnPP-induced cellular***

713   ***aggregates and IκBα-interactome.*** **A**. Venn diagram of IκBα-interacting proteins identified

714   through LC-MS/MS proteomic analyses of IκBα-immunoprecipitates upon IAP and/or ZnPP-

715   induced cellular aggregates from HEK293T and HepG2 cells (*as schematized in Fig. 4*). The

716   complete interaction network of 192 common ZnPP-induced protein aggregates was obtained

717   via STRING analyses with the highest confidence score (≥ 0.9). Network statistics are

718   summarized in the inset. Functional enrichments are annotated with color-lined boxes with the

719   false discovery rates (FDR) included. Of these, the 47 IκBα-interacting proteins are highlighted

720   in yellow. **B**. The corresponding heat map percentile ranked according to the individual fold-

721   enrichment of these 47 IκBα-interacting proteins common to ZnPP-induced aggregates in

722   different experiments. C1-IκBα IP represents mean fold-enrichment from C1-mEmerald-IκBα

723   IAP against C1-Emerald (GFP) background control (n = 3). The other sets represent fold-

724   enrichment of proteins in cellular aggregates at 2 h and 24 h post ZnPP-treatment of HepG2

725   and HEK293T cells relative to corresponding controls. IκBα BAR in +ZnPP/-ZnPP pellet

27

726   aggregates represents fold-enrichment of IκBα-antibody mediated IP of biotinylated proteins in

727   cellular aggregates at 2 h after ZnPP-treatment of HEK293T cells relative to vehicle controls.

728   Proteins previously found in aggregates from other disease models and/or human pathological

729   samples are indicated in red. * Proteins found in liver aggregates from mouse and zebra fish

730   protoporphyric models [13, 36, 37]. [#] Proteins previously found in protein aggregates of

731   pathological samples from human MS and/or Lewy bodies of Parkinson's disease [53, 55]. [&]

732   Proteins previously found in aggregates/ or mislocalized in ALS/FTLD patient samples [54].

733

734   ***Figure 6: Nup153 is an IκBα-interacting protein that is a major target of ZnPP-elicited***

735   ***aggregation.*** **A.** HEK293T cells and HepG2 cells were treated with 10 μM ZnPP for 2 h. Cells

736   were sequentially extracted with Triton and urea buffers. 10 μg of extracts were used for

737   Nup153 and IκBα IB analyses, with actin as the loading control. **B.** Co-immunoprecipitation (Co-

738   IP) of overexpressed IκBα with endogenous Nup153. HEK293T cells were transfected with C1-

739   GFP control vector, or C1-GFP-IκBα vector, or a mock control for 40 h. Co-IP of cytosolic

740   fractions (CER) and nuclear fractions (NER) with GFP-trap followed by Nup153 and IκBα IB

741   analyses, with actin as the loading control, and GAPDH and HistoneH3 (H3) as fractionation

742   controls. **C**. Co-IP of endogenous IκBα with endogenous Nup153. HepG2 cells were treated

743   with 20 ng/ml TNFα for the indicated times followed by co-IP of whole cell lysates with IκBα

744   antibody or control IgG and IB of Nup153 and IκBα with actin as the loading control. **D.** Mouse

745   hepatocytes were either untreated or pretreated with 20 ng/ml TNFα for 1 h and then treated

746   with 10 μM ZnPP or left untreated for the indicated times. Cell lysates were used for IB analyses

747   of Nup153 and IκBα with actin as the loading control. IκBα contents relative to 0 h controls were

748   quantified (Mean ± SD, n = 3, ** P<0.01, * P<0.05). **E.** CMIF analyses. HepG2 cells were treated

749   with vehicle control (Ctrl), or 20 ng/ml TNFα for 0.5 h or 1 h. Some TNFα-treated cells were also

28

750   treated with 10 μM ZnPP 20 min after TNFα-addition and incubated for another 40 min (TNFα +

751   ZnPP 1h). Cells were fixed and stained with anti-p65/anti-rabbit-Alexa-488 IgGs (green) and

752   anti-IκBα/anti-mouse Alexa-647-conjugated IgGs (magenta) and DAPI. Images were obtained

753   under 100X lens. The same slides were then observed under wide-field microscope using 60X

754   lens to quantify the percentage of cells with predominant nuclear p65 accumulation. Cells (>600)

755   were assessed at each experimental condition (Mean ± SD, n = 3).

756

757   **Fig. 7.** *Effects of siRNA knockdown of Nup153 (A) and RanBP2 (B) on TNFα-elicited IκBα*

758   *nuclear import.* HEK293T cells were transfected with control siRNA (Ctrl) or NUP153 siRNA (**A**)

759   or RanBP2 siRNA (**B**) or for 48 h, and then treated with 20 ng/ml TNFα for the indicated times.

760   Cytosol and nuclear extracts were used for IB analyses. IκBα content was plotted over time. **C**.

761   HEK293T cells were transfected with control siRNA (Ctrl) or NUP153 siRNA for 48 h, and then

762   treated with 10 μM ZnPP for the indicated times. Cell lysates were used for IB analyses. **D**.

763   HEK293T cells were transfected with control siRNA (Ctrl) or RanBP2 siRNA for 48 h, and then

764   treated with 10 μM ZnPP for the indicated times. Cell lysates were used for IB analyses.

765

766   **Fig. 8**. *Effects of of IκBα siRNA knockdown on IκBβ-levels and constitutive (A) and*

767   *ZnPP-induced NFκB-activation (B).* siRNA knockdown and EMSA analyses were conducted

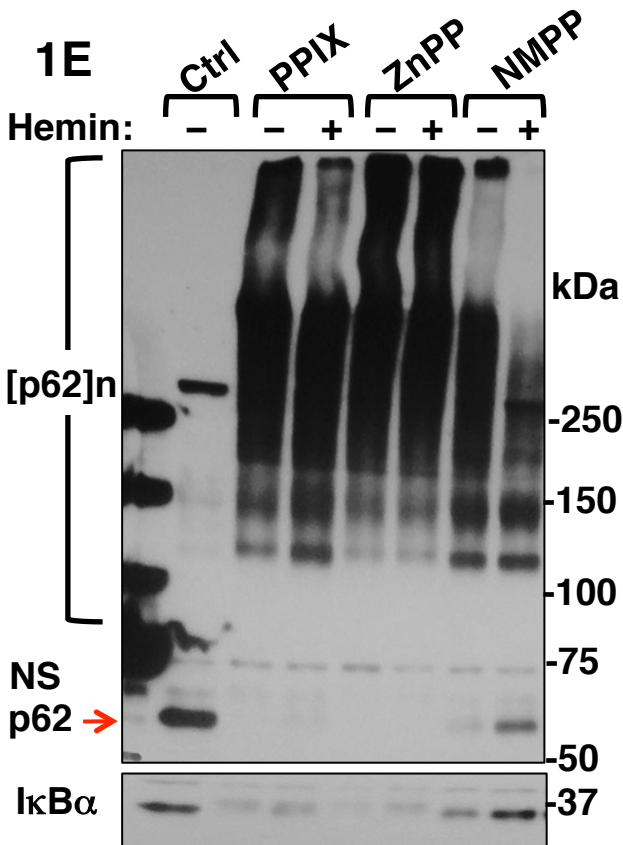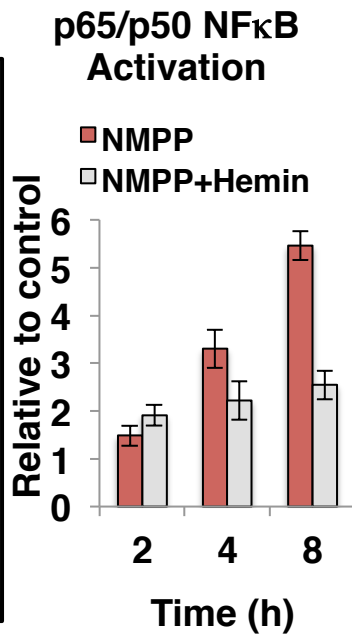768   as detailed in Supplementary Methods. The marked hepatic IκBβ-upregulation coupled with the
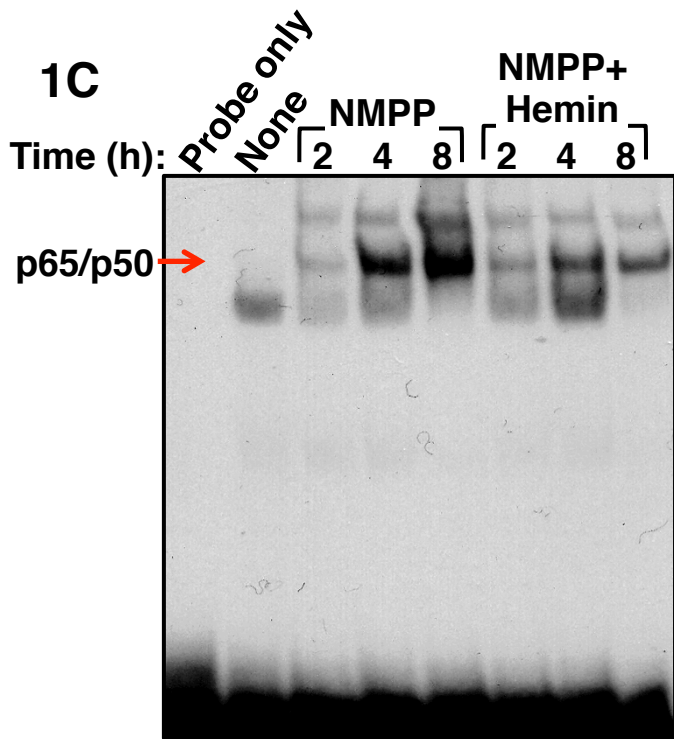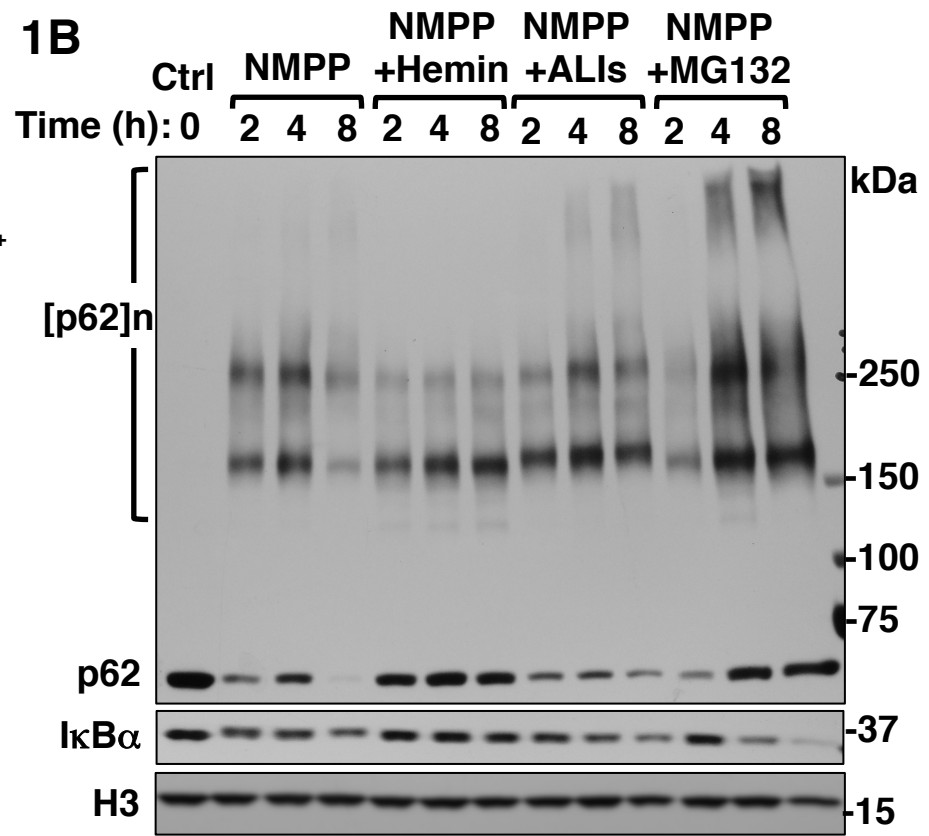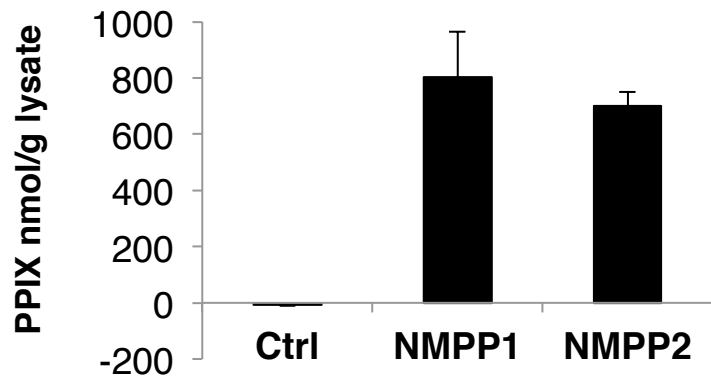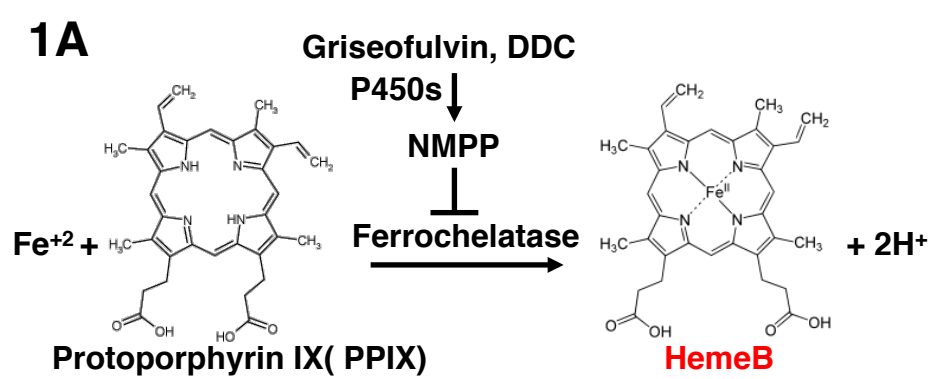
769   relatively less pronounced NFκB-activation observed upon IκBα-siRNA knockdown versus

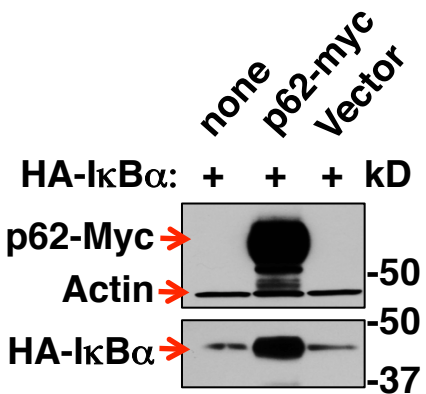770   ZnPP-treatment led to the examination of whether IκBβ was similarly sequestered by ZnPP as

771   IκBα (**C**). Proteolytic inhibitors used as in Fig. 2D. (**D**). qRT-PCR analyses of mRNA from intact

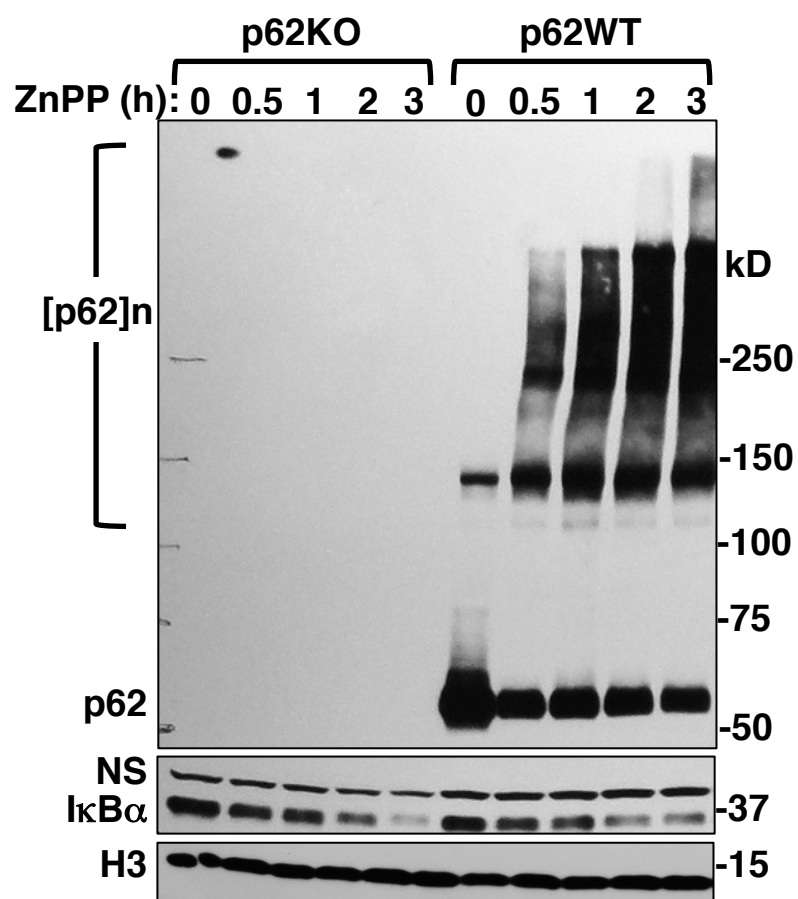772   livers of mice injected i.p., daily with ZnPP (50 μmol/kg) or vehicle controls (Ctrl) for 7 d (Mean,

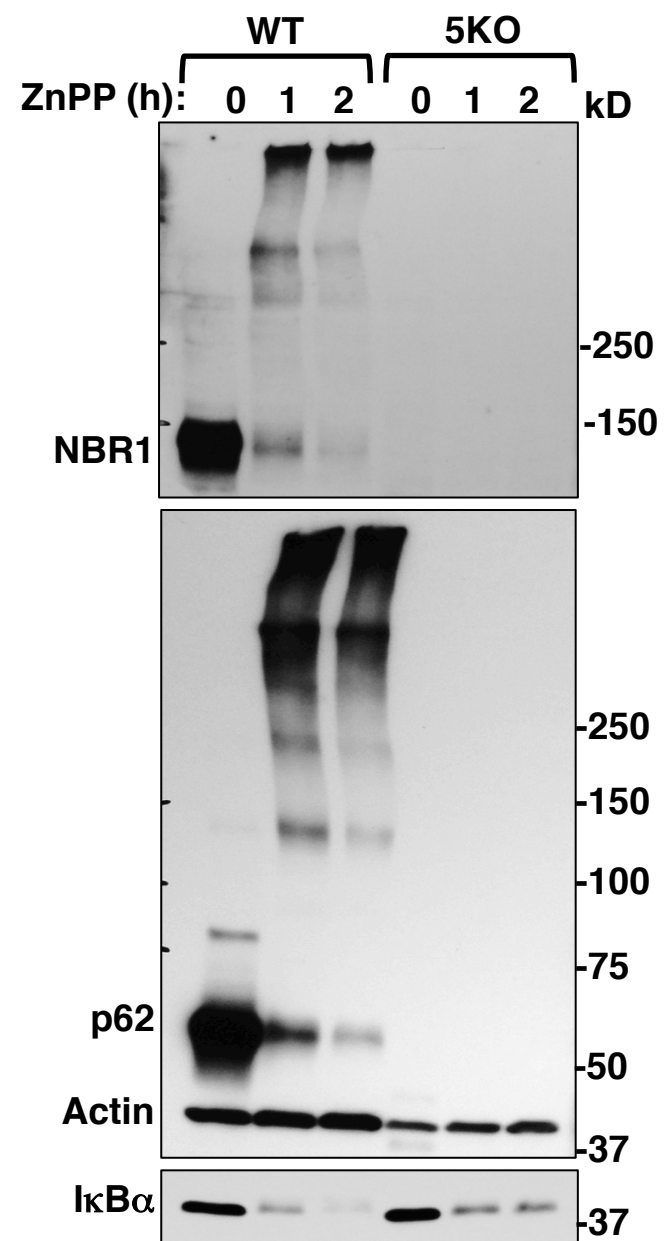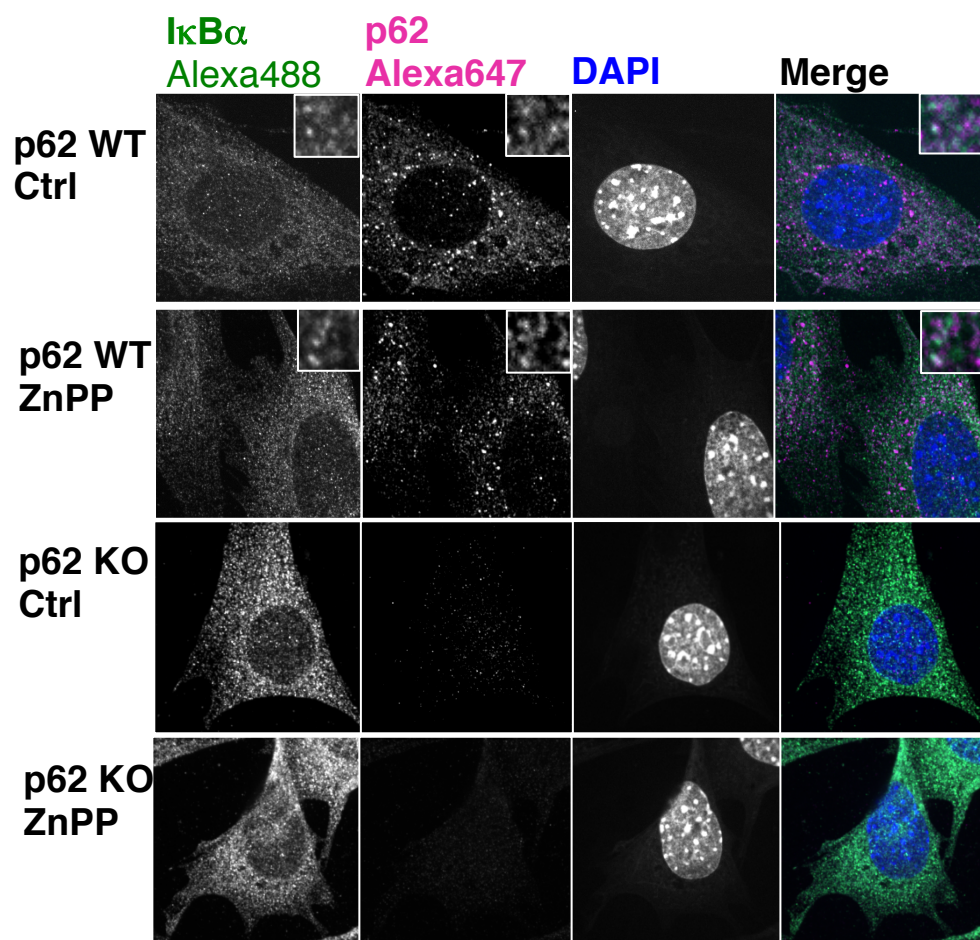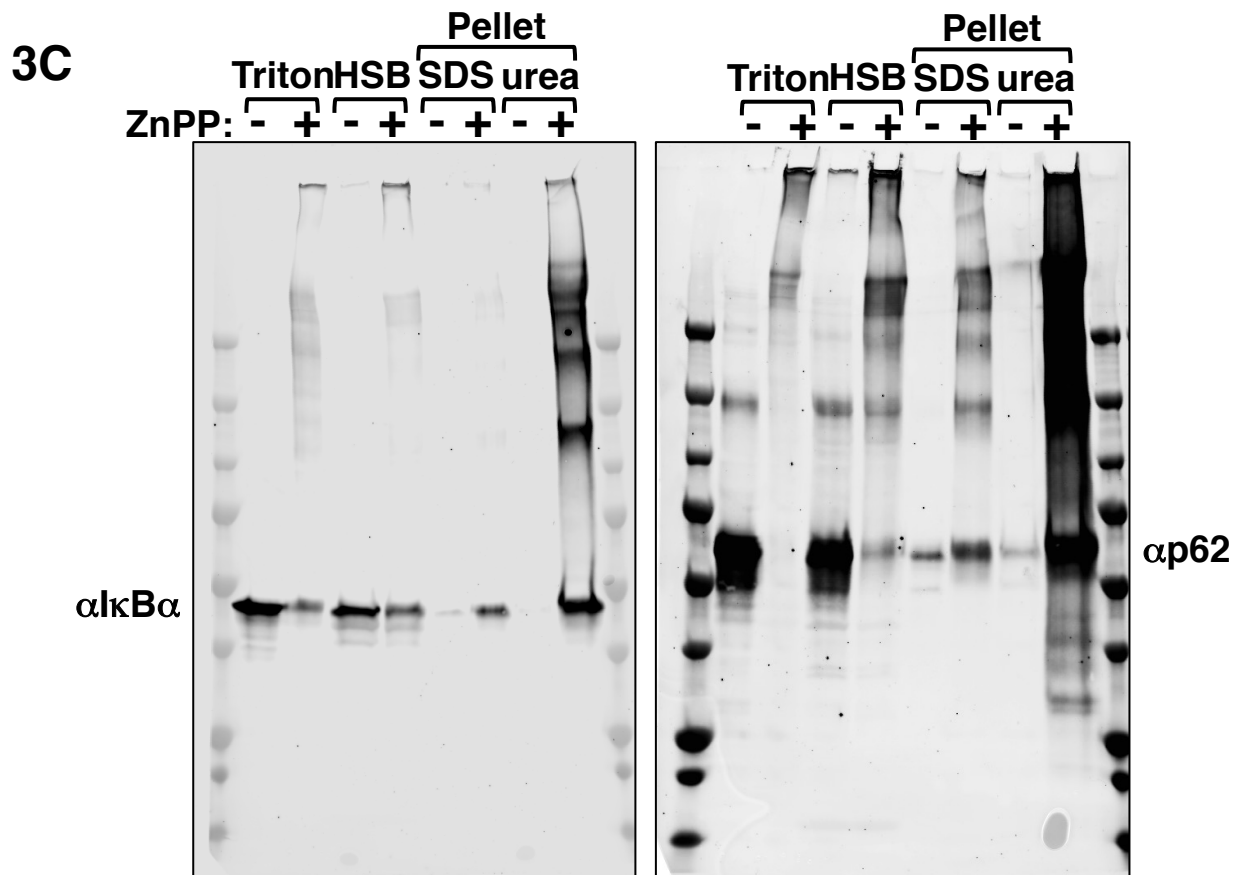773   n = 2). SPP1, osteopontin or secreted phosphoprotein 1.

29

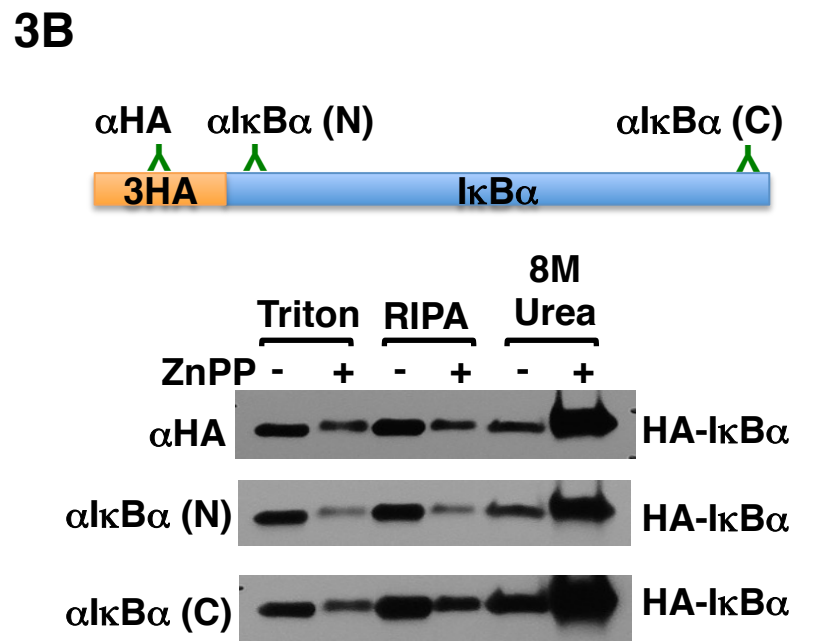**1A**

Griseofulvin, DDC
P450s
↓
NMPP
⊣
Ferrochelatase

Fe^{+2} + Protoporphyrin IX(PPIX) → HemeB + $2H^+$

1B

Ctrl | NMPP | NMPP +Hemin | NMPP +ALIs | NMPP +MG132
Time (h): 0 2 4 8 2 4 8 2 4 8 2 4 8

[p62]n

kDa
— 250
— 150
— 100
— 75

p62
IκBα — 37
H3 — 15

1C

Probe only | None | NMPP | NMPP+Hemin
Time (h): 2 4 8 2 4 8

p65/p50 →

p65/p50 NFκB Activation

- NMPP
- NMPP+Hemin

Relative to control

Time (h): 2, 4, 8

1D

HRI−/− | HRI+/+
NMPP | NMPP
Time (h): 0 2 4 8 0 2 4 8

IκBα
H3

IκBα

- HRI-/-
- HRI+/+

Relative to Control

Time (h)

1E

Ctrl | PPIX | ZnPP | NMPP
Hemin: − | − + | − + | − +

[p62]n

kDa
— 250
— 150
— 100
— 75
— 50

NS
p62 →
IκBα — 37

1F

NER (2μg) | NER (5μg)
Probe | Ctrl | NMPP1 | NMPP2 | PPIX | ZnPP | Ctrl | NMPP1 | NMPP2 | PPIX | ZnPP

p65/p50

Free probe

1G

Ctrl | ZnPP
Time (h): 5 24 | 5 24

IL-6
GAPDH

IL-6 / GAPDH

Time(h): 5 24 | 5 24
Ctrl | ZnPP

2A

none / p62-myc / Vector

HA-IκBα: + + + kD

p62-Myc →

Actin →

—50

HA-IκBα →

—50
—37

2B

p62KO   p62WT

ZnPP (h): 0 0.5 1 2 3   0 0.5 1 2 3

[p62]n

kD

—250

—150

—100

—75

p62

—50

NS
IκBα   —37

H3   —15

2C

WT   5KO

ZnPP (h): 0 1 2   0 1 2 kD

NBR1

—250

—150

—250

—150

—100

—75

p62

—50

Actin

—37

IκBα   —37

2D

IκBα   p62   DAPI   Merge
Alexa488   Alexa647

p62 WT
Ctrl

p62 WT
ZnPP

p62 KO
Ctrl

p62 KO
ZnPP

3A

	Triton	RIPA	Urea Heat	NoHeat
ZnPP:	- +	- +	- +	- +

[p62]n

p62

Actin

HA (HA-IκBα)

H3

3B

αHA   αIκBα (N)   αIκBα (C)

3HA   IκBα

	Triton	RIPA	8M Urea
ZnPP:	- +	- +	- +

αHA   HA-IκBα

αIκBα (N)   HA-IκBα

αIκBα (C)   HA-IκBα

3C

	Triton	HSB	Pellet SDS	urea
ZnPP:	- +	- +	- +	- +

αIκBα

	Triton	HSB	Pellet SDS	urea
ZnPP:	- +	- +	- +	- +

αp62

4A

Emerald

C1-mEmerald
(C1-GFP)

Emerald — IκBα

C1-mEmerald-IκBα
(C1-IκBα)

Transient transfection/HEK293T

Co-IP /GFP-trap

Elute /SDS-PAGE

In-gel digestion/LC-MS/MS

Refine IκBα interactome with an enrichment factor ≥ 2 in all 3 replicates

370 High-Confidence IκBα interactors

4B

Input   Co-IP: GFPtrap

mEmerald-IκBα

mEmerald

4C

RELA/p65
7.193

IKBKAP CHUK
5.923   5.891

NFKB2/p100
6.310

NFKB1/p105
4.315

NFKBIA/IkBa
3.490

Mean Log2 of Enrichment factor

P-value

4D

HepG2
Ctrl/ZnPP 2h

HEK293T
Ctrl/ZnPP 2h

HepG2
Ctrl/ZnPP 24h

HEK293T
Ctrl/ZnPP 24h

Sequential Extraction/SDS-PAGE

In-gel digestion/LC-MS/MS

Identify proteins enriched ≥ 2 fold in ZnPP induced aggregates

Identified 192 proteins commonly aggregating in HEK293T and HepG2 cells

4E

Triton   RIPA   8M Urea
ZnPP: − +   − +   − +

4F

712   192   197

HEK   HepG2

5A

Protein Processing in ER (FDR 2.78E-09)

Nucleo-cytoplasmic Transport (FDR 2.64E-08)

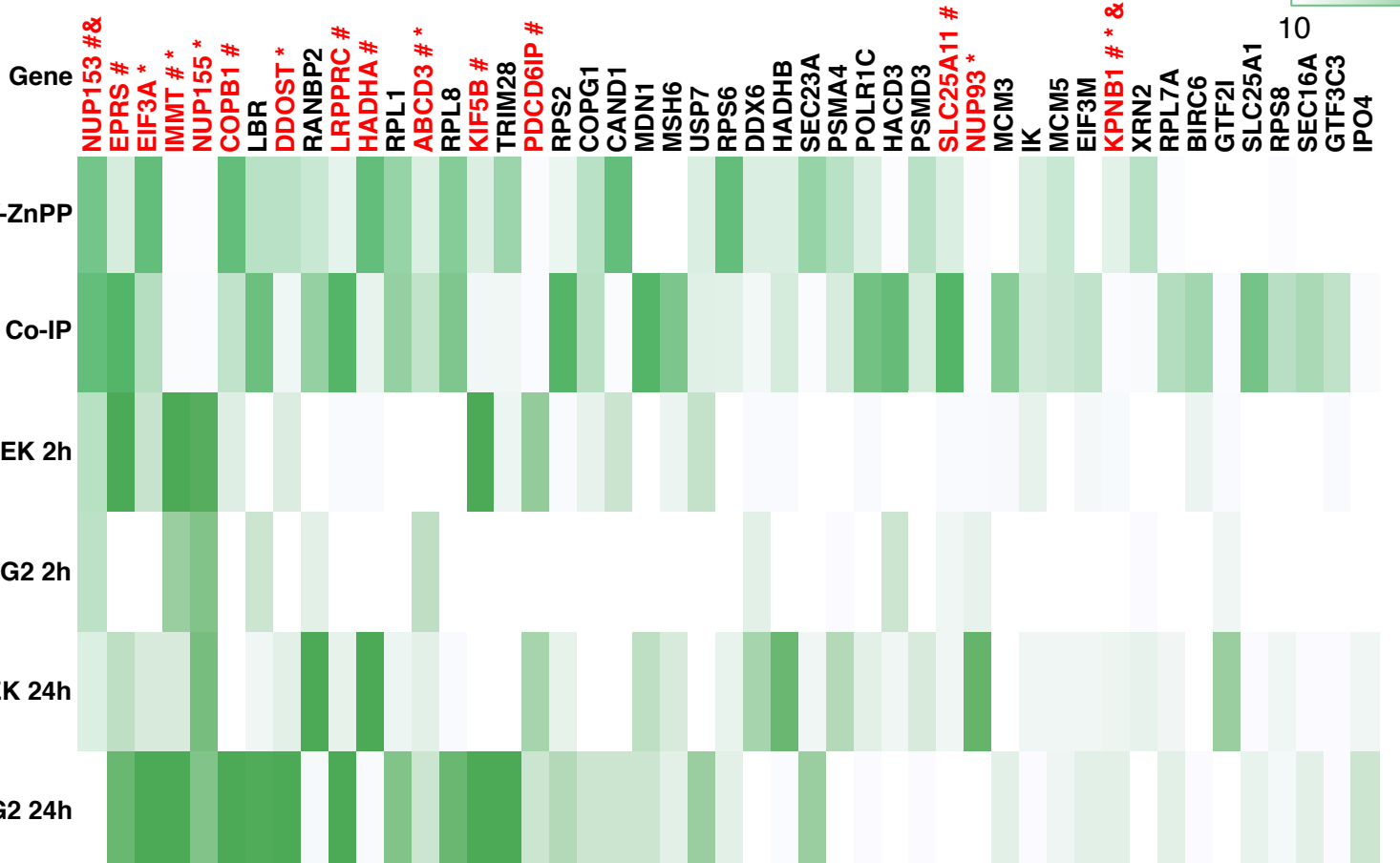**mRNA Processing** (FDR 7.01E-09)
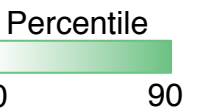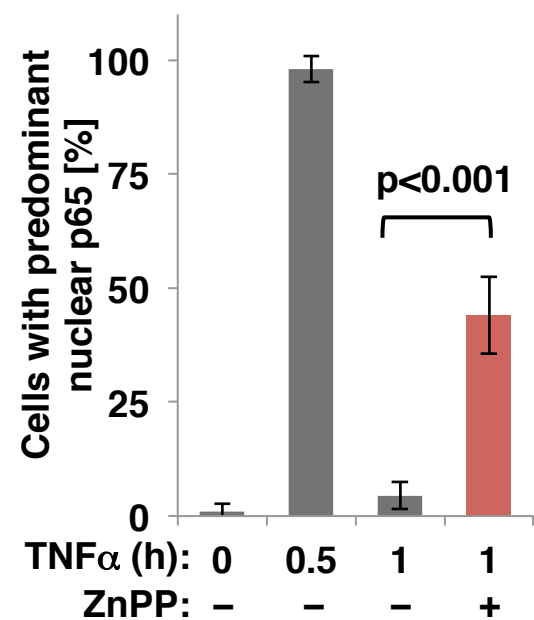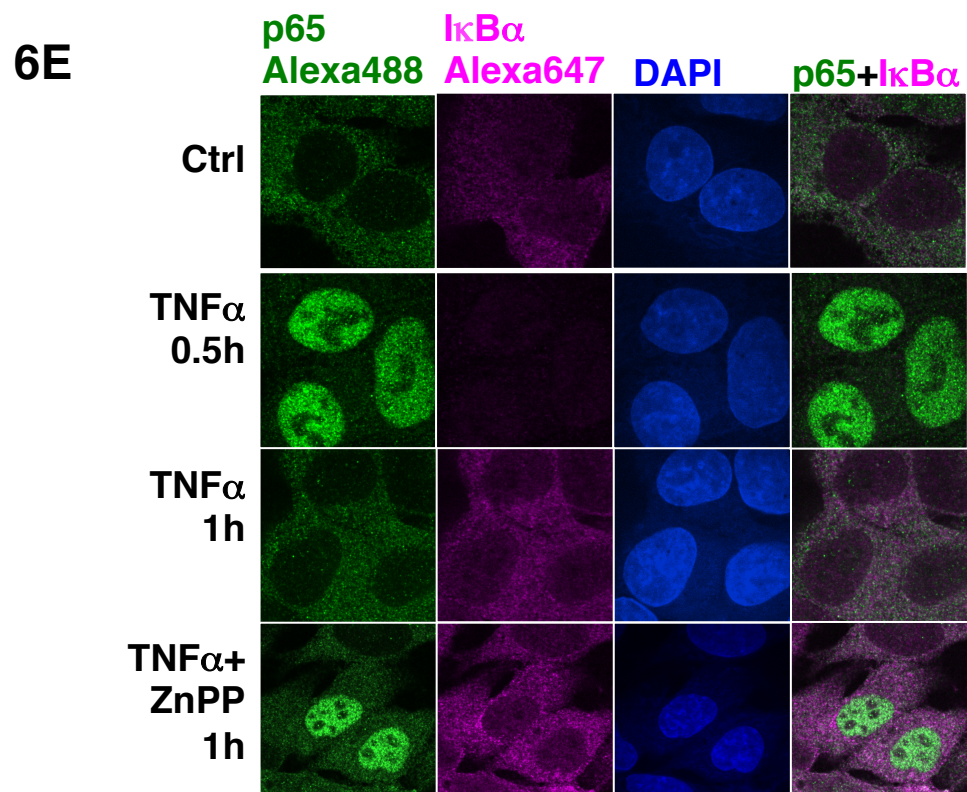
Translational Initiation (FDR 4.66E-14)

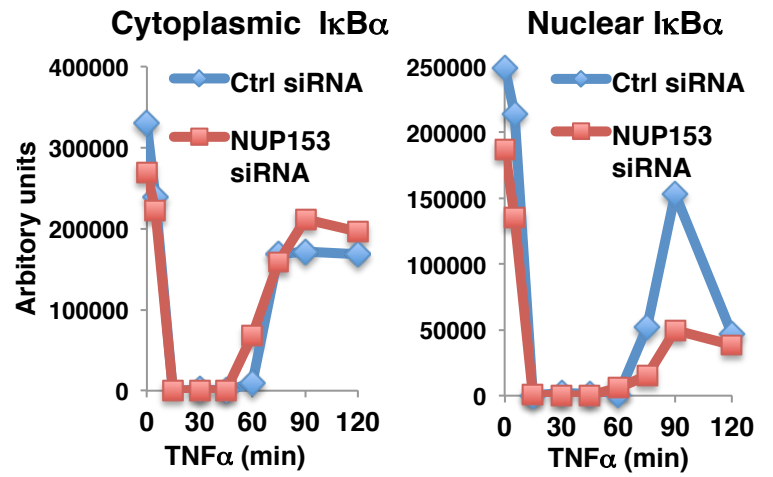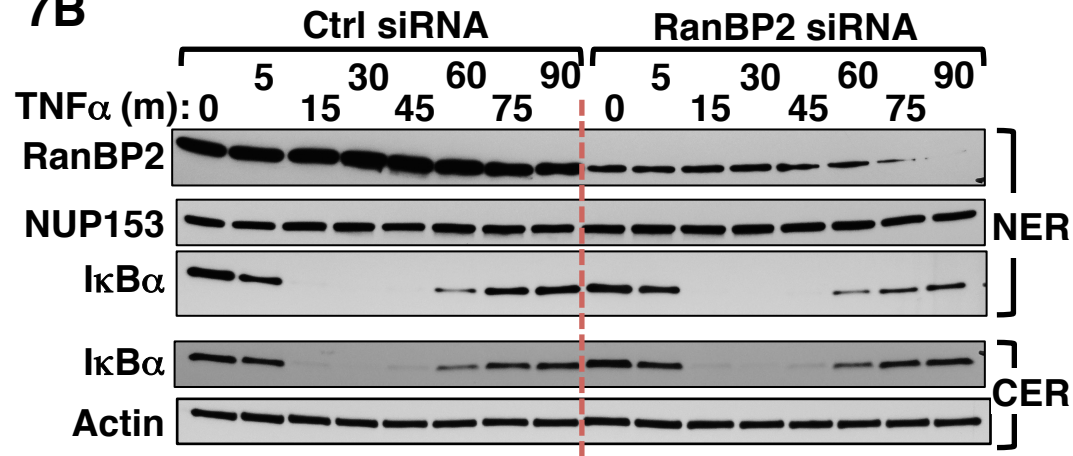| Network Stats	
number of nodes:	189
number of edges:	493
average node degree:	5.22
average local clustering coefficient:	0.527
expected no. of edges:	118
PPI enrichment p-value:	<1E-16

HEK: 595
HepG2: 184
HEK ∩ HepG2: 145
Center: 47
HEK ∩ C1IκBα-IP: 117
HepG2 ∩ C1IκBα-IP: 13
C1IκBα-IP: 193

5B

6A

HEK293T   HepG2
Triton   Urea   Triton   Urea
ZnPP: − + − + − + − +

kDa

250 —

150 —

NUP153

Longer exposure

250 —

150 —

IκBα

37 —

IκBα

37 —

Actin

6B

Input   Co-IP:GFPtrap
CER   NER   CER   NER

Transfection: C1-GFP / GFP-IκBα / Mock

NUP153

IκBα

Actin

GAPDH

H3

6C

Input   Co-IP
           αIκBα   IgG

TNFα (h): 0 1 1.5 2   0 1 1.5 2   0

kDa

150 —

NUP153

50 —

IgG

37 —

IκBα

37 —

Actin

6D

TNFα 1 h pretreat
ZnPP   ZnPP   Ctrl
Time (h): 0 0.5 1 1.5 2   0 0.5 1 1.5 2   0.5 1 1.5 2

αNUP153 (QE5)

NUP153 →

IκBα

Actin

IκBα

ZnPP
TNFα+ZnPP

Relative to contrl — 0.0–1.2

** ** * *

ZnPP (h): 0   1   2

6E

p65 Alexa488   IκBα Alexa647   DAPI   p65+IκBα

Ctrl

TNFα 0.5h

TNFα 1h

TNFα+ZnPP 1h

Cells with predominant nuclear p65 [%]

p<0.001

TNFα (h): 0   0.5   1   1
ZnPP: −   −   −   +

7A

Ctrl siRNA | NUP153 siRNA

TNFα (m): 0 5 15 30 45 60 75 90 120   0 5 15 30 45 60 75 90 120

NUP153
RanBP2   } NER
IκBα

IκBα   } CER
Actin

Cytoplasmic IκBα — Ctrl siRNA, NUP153 siRNA
Nuclear IκBα — Ctrl siRNA, NUP153 siRNA

7B

Ctrl siRNA | RanBP2 siRNA

TNFα (m): 0 5 15 30 45 60 75 90   0 5 15 30 45 60 75 90

RanBP2
NUP153   } NER
IκBα

IκBα   } CER
Actin

Cytoplasmic IκBα — Ctrl siRNA, RanBP2 siRNA
Nuclear IκBα — Ctrl siRNA, RanBP2 siRNA

7C

Ctrl siRNA | NUP153 siRNA

ZnPP (h): 0 1 2   0 1 2

kD

NUP153   −250
          −150

          −250
          −150
          −100
          −75

p62   −50

Actin
IκBα   −37

7D

Ctrl siRNA | RanBP2 siRNA

ZnPP (h): 0 1 2   0 1 2

kD

RanBP2   −250

          −250
          −150
          −100
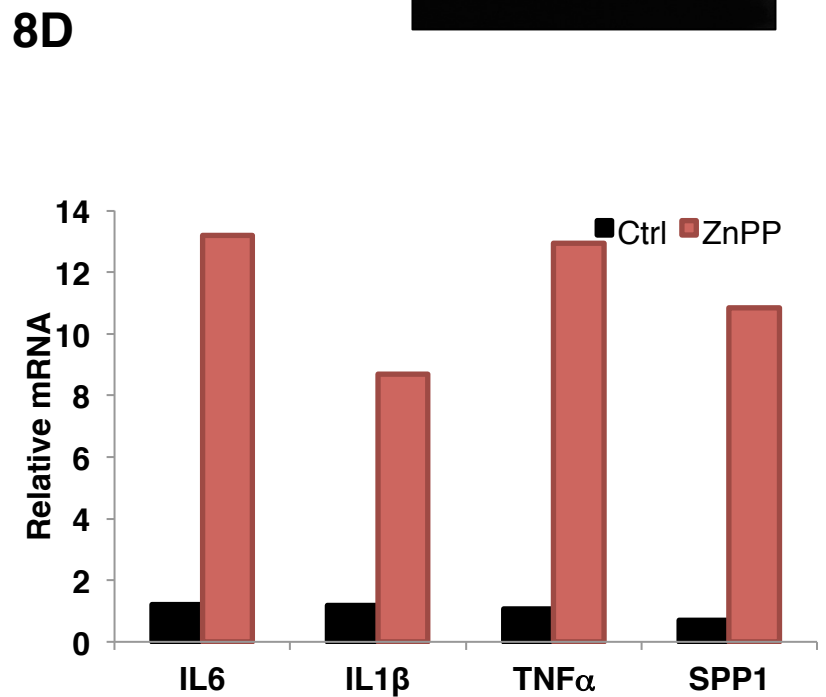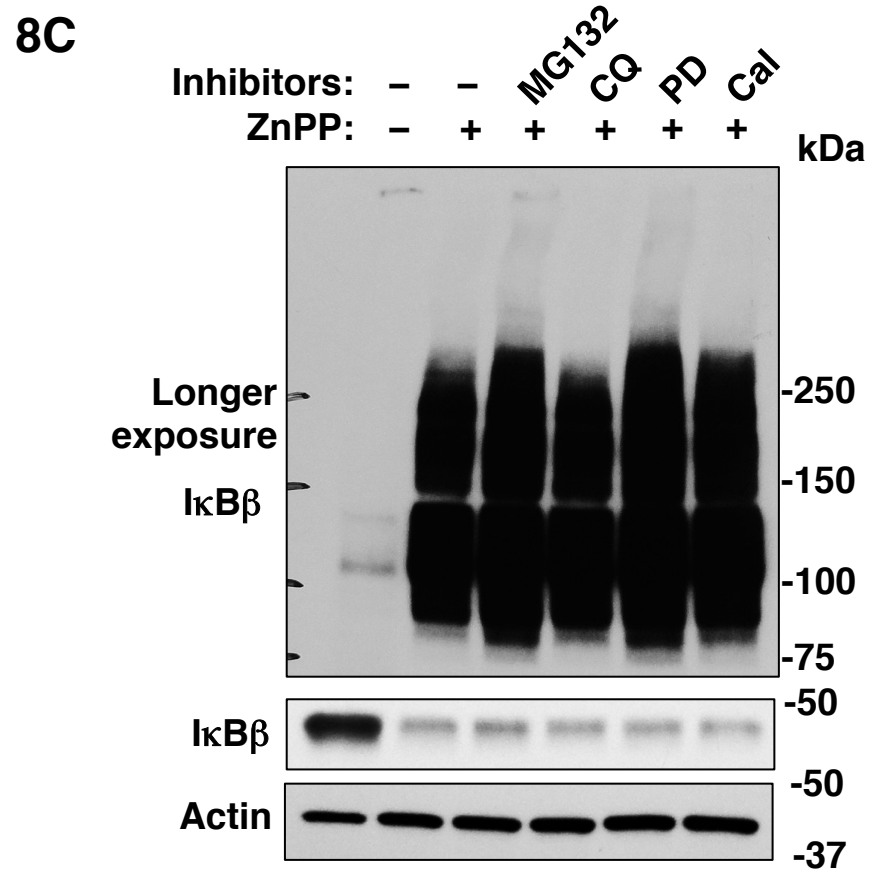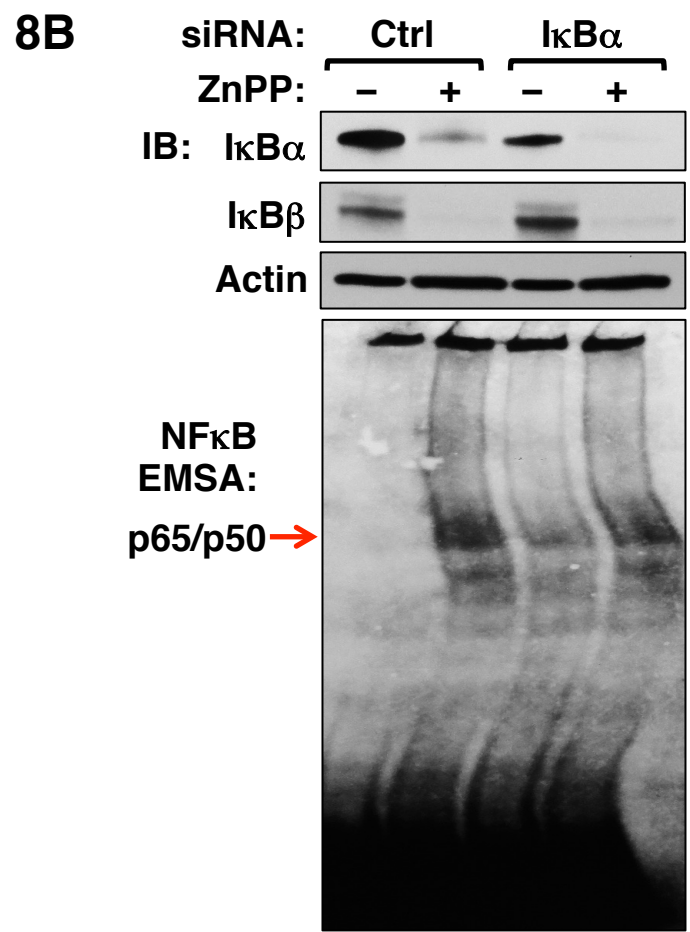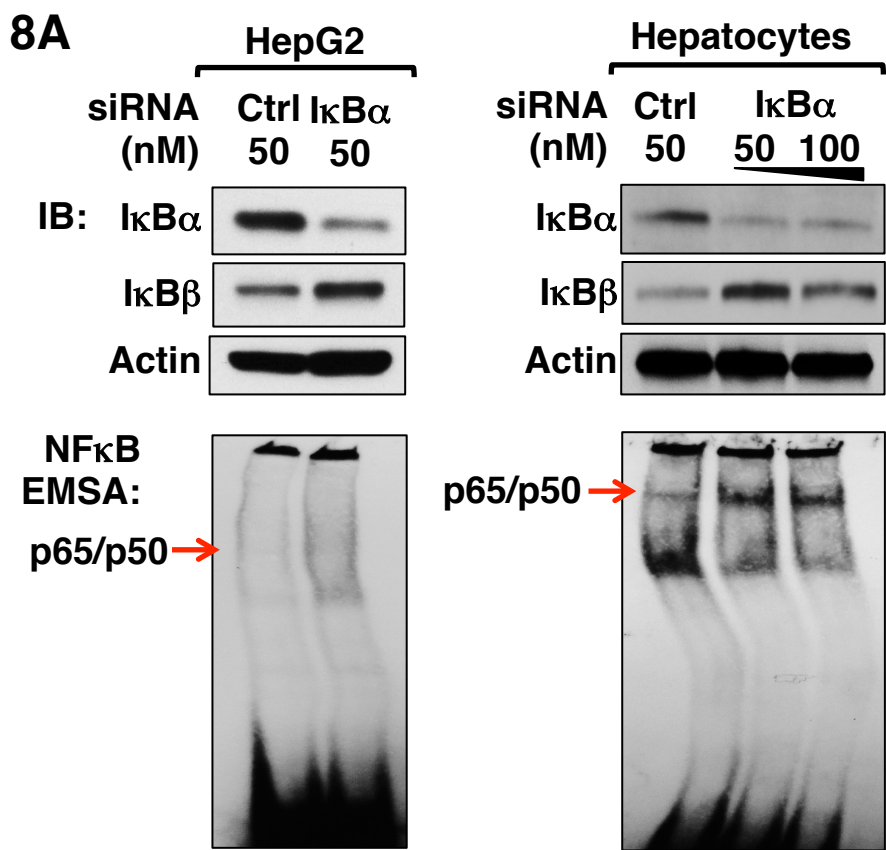   −75

p62   −50

Actin   −37
IκBα   −37

Supplementary Results & Discussion:

Results:
NMPP-elicited IκBα-*loss is independent of HRI eIF2α-kinase activation:* The concurrence of IκBα loss and NF-κB activation and its attenuation by hemin-treatment led us to consider whether NMPP-elicited heme depletion was responsible for these findings. Conceivably, heme depletion could activate the hepatic heme-sensor HRI eIF2α kinase resulting in the translational suppression of IκBα and consequent NF-κB-activation. Similar IκBα translational suppression and consequent NF-κB-activation has been documented upon specific activation of the other cellular eIF2α kinases [1, 2]. Indeed, in parallel with IκBα-loss (Fig S2A), NMPP treatment increased the relative ratio of phosphorylated eIF2α (eIF2αP) over basal eIF2α levels, indicating HRI activation [3]. However, NMPP-treatment of cultured hepatocytes from cultured hepatocytes from HRI WT (HRI$^{+/+}$) and knockout (KO; HRI$^{-/-}$) mice elicited a comparable IκBα, excluding causal HRI activation (Fig. 1D).

ZnPP-elicited IκBα-*loss is independent of autophagy, calpain-mediated proteolysis and reactive oxygen species (ROS):* Given the reported IκBα-degradation via ALD [4] and the known p62-role in this process [5], we conclusively excluded ALD in this process by documenting that knockout of the essential autophagic gene ATG5 failed to mitigate ZnPP-elicited IκBα loss (Fig. S2B). Because of possible calpain-mediated IκBα-degradation [6, 7], we also excluded its involvement by documenting that knockout of the calpain-degradation pathway (capn4 KO MEF cells) failed to abrogate ZnPP-elicited IκBα loss (Fig. S2C). These findings coupled with those with ALD and calpain inhibitors (Fig. 2D) revealed that neither ALD nor calpain degradation played any role in this IκBα loss. Furthermore, through the use of various ROS-quenchers (Fig. S3), we excluded any possible PPIX-photoactivation and consequent ROS-mediated oxidative stress in this IκBα-loss [8].

Discussion:
The precise mechanism of the ZnPP-elicited IκB-protein sequestration is currently unknown, but some plausible mechanisms are presented. The fact that newly synthesized IκBα is particularly vulnerable to such ZnPP-sequestration implicates the involvement of its intrinsic disordered domains at a stage when it has not yet reached its mature folded state.

In search of clues on potential ZnPP-mechanisms of protein sequestration, we sought the advice of various internationally recognized expert heme/porphyrin chemists and biochemists and porphyria experts on the possible mechanistic causes. Not a single investigator was aware that ZnPP caused protein aggregation, much less IκBα-sequestration. ZnPP (along with SnPP), is actually believed to be safe and is recommended for the treatment of neonatal jaundice due to its effective inhibition of microsomal heme oxygenase (HO1), the key rate-limiting enzyme in the conversion of heme to biliverdin [9]. However, *in vivo*, overproduction of protoporphyrin IX (PPIX) due to defects in ferrochelatase (congenital erythropoietic protoporphyria) or PPIX-overproduction in X-linked protoporphyria, as well as iron-deficiency or lead-poisoning induced anemias (that can exhaust iron-stores) leads to ZnPP-generation [10-12]. Our findings suggest that under these conditions, these patients may easily succumb to ZnPP-elicited IκBα-sequestration and consequently unabated hepatic NF-κB-elicited activation of cytokines and chemokines. We believe, these findings would for the first-time alert physicians of this pathological potential of ZnPP and are clinically relevant not just in MDB-inducing diseases but also in clinical protoporphyrias and iron-deficiency/lead-induced anemias.

Supplementary methods

Contact for reagent and resource sharing

Information and requests for resources and reagents should be directed to M. A. Correia (almira.correia@ucsf.edu).

Experimental model details

Animal studies:

C57BL/6 wild type male mice (8-12-week old) purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were fed a standard chow-diet and maintained under a normal diurnal light cycle. All animal experiments were carried out strictly by protocols specifically approved by the UCSF/Institutional Animal Care and Use Committee (IACUC) and its care and use of laboratory animal guidelines. For the ZnPP-treatment, ZnPP was dissolved in sterile DMSO at 100 mM, and then diluted 1:10 (v:v) with sterile 7.5% BSA to a final concentration of 10 mM. Mice were weighed and then injected daily with either ZnPP (50 µmol/kg, i.p.), or vehicle (controls) (10% DMSO in 7.5% BSA) for 7 days. Liver samples were collected and frozen at -80°C until RNA isolation.

Cell culture:

All cells were grown at 37°C with 5% CO_2 in a humidified incubator. C57BL/6 wild type male mice (8-12-week old) purchased from the Jackson Laboratory (Bar Harbor, ME) were used for primary hepatocyte preparation. Hepatocytes were isolated by *in situ* collagenase perfusion and purified by Percoll-gradient centrifugation by the UCSF Liver Center Cell Biology Core, as described previously [13]. Fresh primary mouse hepatocytes were cultured on Type I collagen-coated 60 mm Permanox plates (Thermo Scientific, Grand Island, NY) in William's E Medium supplemented with 2 mM L-glutamine, insulin-transferrin-selenium, 0.1% bovine albumin Fraction V, Penicillin-Streptomycin and 0.1 µM dexamethasone. Cells were allowed to attach for 4 to 6 h and then overlaid with Matrigel. From the 2nd day after plating, the medium was replaced daily, and cells were further cultured for 4-5 days with daily light microscopic examination for any signs of cell death and/or cytotoxicity. On day 5, some cells were treated with 30 µM NMPP (dissolved in DMSO), 10 µM PPIX (dissolved in DMSO with sonication) or 10 µM zinc (II)-PPIX (ZnPP, dissolved in DMSO and complexed with bovine serum albumin (BSA) at a molar ratio of 4:1 to keep it solubilized in the medium) for various times as indicated (Results). In some cases, hepatocytes were pretreated with various inhibitors as indicated (Results) for 1 h before treatment with NMPP, PPIX or ZnPP.

HepG2 and HeLa cells were cultured in minimal Eagle's medium (MEM) containing 10% v/v fetal bovine serum (FBS) and supplemented with nonessential amino acids and 1 mM sodium pyruvate. HEK293T and MEF cells were cultured with Dulbecco's Modified Eagle high glucose medium (DMEM) containing 10% v/v FBS. For transfection experiments, cells were seeded onto 6-well plates, and when cells were 60% confluent, each cell well was transfected with 3 µg plasmid DNA complexed with TurboFect transfection reagent for HEK293T cells and X-tremeGENE HP transfection reagent for HepG2 cells according to the manufacturers'

instructions. At 40-72 h after transfection, cells were either treated as indicated or directly harvested for assays.

Method details

Hepatic PPIX content: PPIX content was determined using the intrinsic PPIX fluorescence as described [14]. Briefly, 50 µl of cell lysates were first extracted with 400 µl of EtOAc-HAc (3:1) and then re-extracted with another 400 µl of EtOAc-HAc. The extracts were pooled and reextracted with 400 µl of 3 M HCl. After centrifugation, the aqueous phase was recovered for fluorescent determination in a SpectrumMax M5 plate reader at an excitation 405 nm. The intensity at emission 610 nm was quantified relative to a standard curve prepared with known concentrations of pure PPIX.

Plasmids: The primers, templates, vectors and restriction enzymes (RE) used for constructing plasmids used in this study are summarized below:

Plasmid	Template	PCR primers (restriction sites underlined)	Vector	RE
C1-Emerald-IκBα	pCMV-3HA-IκBα	GCTC<u>AAGCTT</u>CGttccaggcggccgagcg Ggaggccagcgtctgacgtta**tga**<u>CCCGGG</u>AT CC	C1-Emerald	*HindIII Smal*
pcDNA6-p62-Myc	cDNA clone (IMAGE:4298142)	<u>AAGCTT</u>**atg**gcgtcgctcaccg gcatcccccgccgttg<u>CTCGAG</u>	pcDNA6.1 Myc/His$_6$	*HindIII XhoI*
pcDNA3-NBR1	cDNA clone (IMAGE: 2989212)	TAGA<u>CTCGAG</u>GCCACC**atg**gaaccacag gttactc ctggtacagccaacgctatt**tga**<u>CTCGAG</u>TCTA	pcDNA3.1	*XhoI KpnI*

Electrophoretic mobility shift assay (EMSA): Nuclear fractions of cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents. EMSA were performed using LightShift Chemiluminescent EMSA Kit. Briefly, 2-5 µg of nuclear extracts were incubated with 1 µl of biotin-labeled NFkB DNA probes in binding buffer [10 mM Tris, pH 7.5, 50 mM KCl, 1 mM DTT, 5 mM MgCl$_2$, 0.2 mM EDTA, 5% glycerol, 0.5% NP-40, 1 µg of poly dI-dC]. The final volume of the mixture was adjusted to 20 µl and incubated at room temperature for 30 min, and then mixed with 5 µl of loading buffer for loading onto 5% TBE Polyacrylamide Gel (Biorad, Hercules, CA). The gel was run at 120 V until the dye reached the gel bottom, and then transferred to Hybond-N+ positively charged nylon membrane (GE Life Sciences, Marlborough, MA). The NF-κB complex shifted probes were detected by blotting with HRP-coupled streptavidin. For super-shift EMSA, the binding mixture (in the absence of the biotin-labeled probes) was first incubated with p65 antibody for 20 min on ice, and then the biotin-labeled probes were added and further incubated at room temperature for another 30 min before gel loading.

RNA isolation and semi-quantitative RT-PCR: Total RNA was extracted using RNeasy Mini Kit according to the manufacturers' instructions. Total RNA (2 µg) was used to perform reverse transcription using SuperScript VILO Master Mix in a 20 µl-reaction. Reverse transcribed first strand cDNA (1 µl) was used in PCRs.

Western Immunoblotting (IB) Analyses: For Western IB analysis, whole-cell extracts were prepared with Cell Lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin and supplemented with 10% glycerol and protease/phosphatase inhibitor cocktail. Cell lysates were sonicated for 10s and then cleared by centrifugation at 4°C in a tabletop centrifuge at the highest speed for 10 min. Protein concentrations were determined by BCA assay and equal amounts of proteins were separated on 4-15% Tris-Glycine eXtended (TGX) polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes (Biorad, Hercules, CA) for IB analyses. The antibodies used are listed in Key Resources Table.

Co-Immunoprecipitation (Co-IP) analyses: Whole-cell extracts were prepared as described above. Cell lysates (1 mg) were then incubated with indicated antibodies (2 μg) or control IgGs at 4°C overnight. Antibody-antigen complexes were then captured by protein G Dynabeads at room temperature for 1 h, and then eluted by heating at 95°C for 10 min in 2X SDS-loading buffer. Eluates were subjected to IB analyses as described above.

Sequential solvent extraction of cell lysates: Cells were harvested in cell lysis buffer as described above and the cell lysates were cleared by centrifugation at 14,000g. The pellet was then solubilized in RIPA buffer supplemented with 0.1% SDS, 10% glycerol and protease/phosphatase inhibitor cocktail with sonication followed by centrifugation at the highest speed for 10 min. The resulting pellet was then solubilized with sonication in urea/CHAPS buffer containing 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, and 30 mM DTT and supplemented with protease/phosphatase inhibitor cocktail. High salt buffer (HSB)-extraction (Fig. 4C) was carried out as described previously [15]. Briefly, cells were first harvested in cell lysis buffer containing 1% Triton as described above, the resulting pellet was then suspended in a cell lysis buffer supplemented with 1.5 M KCl (HSB) with sonication. Upon sedimentation, the resulting pellet was solubilized in Laemmli buffer containing 4% SDS or in urea/CHAPS buffer as described above.

Confocal Immunofluorescence microscopy (CIFM): Cells were grown on collagen-coated glass coverslips and treated as indicated. Cells were fixed with 4% formaldehyde for 20 min at room temperature followed by methanol at -20°C for 1 min. After that, cells were rinsed with PBS and blocked for 1 h with 10% normal goat serum in PBS /0.1% Tween at room temperature, and then stained with indicated primary antibodies at 4°C overnight. Cells were then washed in PBS 0.1% Tween three times and then stained with secondary antibodies for 1 h at room temperature. Cells were further washed three times in PBS 0.1% Tween and then mounted using ProLong Diamond Antifade Mountant with DAPI nuclear stain (Molecular Probes, Grand Island, NY). The following secondary antibodies were applied: Goat anti-rabbit IgG Alexa Fluor 488 (Invitrogen, Grand Island, NY), anti-mouse IgG Alexa Fluor 647 (Cell Signaling Technology, Danvers, MA). These particular fluor dyes were selected to circumvent any specific interference from ZnPP intrinsic fluorescence. Images were taken with a Nikon Yokogawa CSU-22 Spinning Disk Confocal Microscope using a Plan Apo VC 100x/1.4 oil lens or on a Nikon high-speed wide-field Andor Borealis CSU-W1 spinning disk confocal microscope using a Plan Apo VC 60x/1.4 oil lens. Images were processed using ImageJ software. For quantification, at least 600 cells at each condition were evaluated. Statistical significance was tested using two-sided unpaired Student's t-test.

Immunoaffinity purification (IAP): We employed high-affinity alpaca Nanobody crosslinked beads (GFP-Trap) for IAP, which not only enabled a high-level enrichment of target proteins but

also eliminated IgG contaminants that confound downstream LC-MS/MS analyses. N-terminally mEmerald (GFP)-tagged IκBα (GFP-IκBα) was transiently transfected into HEK293T cells, with mEmerald-transfected cells as background control (C1-GFP). Two wells of HEK293T cells grown on 6-well plates were pooled and lysed in 1 ml lysis buffer supplemented with 10% glycerol, protease/phosphatase inhibitor cocktail and 20 mM N-ethylmaleimide (NEM). Centrifugation-cleared cell lysates were incubated with 50 μl GFP-trap agarose beads at 4°C overnight. Subsequently, GFP-trap beads were collected by centrifugation at 3,000g for 30s and then washed 5 times with cell lysis buffer. Co-immunoprecipitated proteins were eluted by incubating beads with 2X Laemmli buffer at 70°C for 15 min. Eluates were then subjected to SDS-PAGE and stained with Coomassie Blue to visualize the bands for subsequent in-gel digestion.

Biotinylation by antibody-recognition (BAR): Traditional approaches for in vivo protein-protein interactions such as co-IP are based on affinity capture of stable protein complexes that will be disrupted under harsh denaturing conditions. By contrast, biotinylation proximity labeling circumvents this limitation by introducing an enzyme to the target protein that can generate distance-constrained reactive biotin molecules to covalently link neighboring proteins, providing a permanent tag that survives purification under harsh conditions for downstream identification [16]. We chose BAR/APEX over BioID (the two popular methods for in vivo biotinylation proximity) because the BAR approach [17] is much more robust as it enables quicker capture of interacting proteins (as the peroxidase mediated biotinylation takes only a few minutes) without the more protracted BioID methodology (requiring 18-24 h tagging time), which could overlook short-term/transient interactants. BAR analyses were performed according to [17]. Briefly, two wells of HEK293T cells grown on collagen-coated 6-well plates were first treated as indicated and then fixed with 4% formaldehyde for 10 min at room temperature and permeabilized for 7 min with 0.5% Triton-X in PBS. After rinsing with PBS, cells were incubated with 0.5% H_2O_2 for 10 min. After rinsing with PBS, cells were then blocked for 1 h with 10% normal goat serum in PBS/0.1% Tween, and then stained with IκBα antibody (mouse monoclonal, 44D4) at a 1:100 dilution in 1% normal goat serum in PBS/0.1% Tween at 4°C overnight. Negative control staining with no antibody was also performed. Cells were then washed in PBS 0.1% Tween for over 1 h with at least 5 buffer changes and then stained with secondary poly-HRP-conjugated goat anti-mouse IgGs (from Biotin XX Tyramide SuperBoost™ Kit) at a 1:1000 v:v dilution in 1% normal goat serum in PBS/0.1% Tween for 1 h. Cells were further washed for over 2 h in PBS 0.1% Tween with at least 5 buffer changes. After that, cells were labeled with biotin using Biotin XX Tyramide SuperBoost™ Kit following product instructions. Briefly, cells were pre-incubated with 300 ml of reaction buffer containing biotin-XX-tyramide (Biotin-Phenol) for 15 min; H_2O_2 was then added to obtain a final concentration of 0.5 mM and incubated for another 5 min. Negative controls without H_2O_2 were also included. The labeling reaction was then stopped by quickly exchanging the reaction solution with 1 ml of 500mM sodium ascorbate. Cells were washed 3 times with PBS and then lysed in RIPA buffer supplemented with 2% SDS and boiled for 1 h to reverse formaldehyde crosslinking. The cell lysates were cleared by centrifugation at 14,000g, the supernatants were used as whole cell lysates (WCL). The resulting pellet was further solubilized in urea/CHAPS buffer as described above. Aliquots from both fractions were used for streptavidin-HRP blot (Invitrogen, Grand Island, NY, #S911), to characterize the specificity and efficiency of BAR. The remaining WCL and urea/CHAPS fractions were separately diluted 1:2 (v;v) in RIPA buffer for streptavidin (SA) pull-down. SA pull-downs were performed according to [18]. Briefly, 50 μl of streptavidin magnetic beads were added to the lysates to incubate at 4°C overnight on a rotator. Beads were then washed twice with RIPA buffer, once with 1 M KCl, once with 2 M urea in 20 mM Tris base, and lastly, twice with RIPA buffer. Biotinylated proteins were then eluted by incubating beads in 40 μl of 2X Laemmli buffer

supplemented with 2 mM biotin at 95°C for 5 min. Eluates were then subjected to SDS-PAGE and stained with Coomassie Blue to visualize the bands for subsequent in-gel digestion. For SA pull-down from urea/CHAPS fractions, the last 2 washes were performed using 100 mM ammonium bicarbonate (ABC) before proceeding with on-beads digestion.

Mass spectrometry (MS): For in-gel digestion, each gel lane was sliced into 8-10 sections and processed separately using a standard in-gel digestion procedure [19, 20]. Briefly, each gel piece was reduced and alkylated, and then digested overnight with 300 ng of Trypsin/Lys-C Mix (Promega, Madison, WI). For on-bead digestion after SA pull-down, beads were first resuspended in 6 M urea in 100 mM ABC, and reduced by adding final 10 mM DTT and incubation at 37°C for 30 min. The samples were then alkylated by adding final 15 mM iodoacetamide with incubation at room temperature in the dark for 30 min. Trypsin/Lys-C Mix (100 ng) was then added and the mixture incubated for 4 h at 37°C. The mixture was then diluted 1:6 (v:v) with 100 mM ABC to reduce the urea concentration to 1 M and then further incubated at 37°C overnight. The resulting peptide mixture was desalted using C18-Zip tips (Millipore, Hayward, CA), speed-vacuumed to dryness and suspended in 0.1% formic acid for injection into an LTQ-Orbitrap Velos mass spectrometer (Thermo Fisher, Grand Island, NY) coupled to a nano-Acquity UPLC (Waters, Milford, MA) with an EASY-Spray column (75 μm x 15 cm column packed with 3 μm, 100 Å PepMap C18 resin; Thermo Scientific, Grand Island, NY). Each sample was separated on the column following a 90-min solvent gradient (Solvent A: Water/0.1% formic acid; Solvent B: Acetonitrile/0.1% formic acid) and the mass spectrometer was operated under high-energy collisional dissociation (HCD) mode. Peak lists were extracted using PAVA, an in-house software developed by UCSF Mass Spectrometry facility. Peak lists from 8-10 mass spectometric fractions under each experimental condition were pooled to search against the Swissprot human database (SwissProt.2015.12.1; 20194/549832 entries searched) using ProteinProspector (version 5.19.1; http://prospector.ucsf.edu/prospector/mshome.htm) [21]. A fully randomized database was used to estimate false discovery rates (FDR) [22]. Score thresholds were chosen at <1% FDR at the peptide level. ProteinProspector search parameters were as follows: Tolerance for precursor and product ions were 20 ppm and 25 ppm; a maximum of 1 missed cleavage of trypsin was allowed; carbamidomethylation of cysteine was set as a fixed modification; variable modifications include: N-terminal Met loss and/or acetylation, Met oxidation, peptide N-terminal Gln to pyroGly conversion; number of variable modifications was 2. Reporting thresholds were as follows: Minimum score of protein: 22.0; minimum score of peptide: 15.0; maximum E value of protein: 0.01; maximum E value of peptide: 0.05.

Mass spectral data processing: To obtain a high-confidence IkBa-interactome, three independent IAP-MS experiments were performed, (from cell transfection, IAP to MS protein identification), each consisting of three biological replicates (three wells each individually transfected). The identified proteins were strictly filtered as follows: 1) From each LC-MS/MS run, only proteins with two or more peptides identified were utilized for further analyses; 2) when some unique peptides matched to multiple protein isoforms, only the isoform with the highest number of matched peptides was reported; 3) only proteins that were immunoaffinity purified with mEmerald- IκBα in all three replicates were kept for further analyses. After filtering, an enrichment ratio of total spectral counts for each protein in mEmerald-IκBα IAP over GFP (C1-mEmerald) background control was calculated as described [23]. Proteins that were not detected in the background control were artificially assigned 0.5 spectral counts, so that an enrichment fold could be calculated. Only proteins that were enriched ≥ 2-fold in all three

replicates were considered high-confidence interactors. Mean ratio and p-value from three replicates were then obtained. p-values were obtained using a paired Student's t-test with total spectral counts from GFP-control compared to mEmerald-IκBα IAP values. To characterize the aggregate-proteome, proteins in high molecular mass (HMM)-regions and urea/CHAPS-solubilized fractions from HEK293T and HepG2 cells were similarly filtered and those in ZnPP-treated and control samples were directly compared. Identified proteins were filtered as described above. Overlap analyses were performed using the Venn Diagram tool (http://bioinformatics.psb.ugent.be/webtools/Venn/).

Network analysis of aggregated proteins: 192 commonly found proteins in ZnPP-induced aggregates from both cell lines were searched against a STRING database version 10.0 [24] for protein-protein interactions. We used the highest confidence score ($\geq$ 0.9) to obtain a protein-interaction network. The network was exported to Cytoscape software [25] for pathway annotation and visualization.

siRNA-knockdown analyses: HEK293T cells were transfected using DharmaFECT 4 Transfection Reagent, with 10 nM siRNA specific for RanBP2 or NUP153. Treatments were performed 48 h after transfection. HepG2 cells were transfected using DharmaFECT 4 Transfection Reagent with 50 nM siGENOME Human NFKBIA siRNA-Smartpool or control non-targeting siRNA. Primary mouse hepatocytes, were transfected using DharmaFECT 4 Transfection Reagent with 50-100 nM siGENOME Nfkbia siRNA-SMARTpool or control siRNA non-targeting siRNA, 16 h before overlay with Matrigel. Cells were harvested 72-96 h after transfection.

Bioinformatic Analyses

GRAVY scores estimating average hydrophobicity was calculated using Kyte and Doolittle hydropathic analyses (http://web.expasy.org/protparam/) [26]. Positive GRAVY scores suggest more hydrophobicity. Proteins with long intrinsically disordered regions was predicted using SLIDER method (http://biomine.cs.vcu.edu/servers/SLIDER/) [27]. This computational method is based on the physicochemical properties of amino acids, sequence complexity, and amino acid composition, a SLIDER score >0.538 suggests a high likelihood that the protein contains a long (>30 residues) disordered segment.

Quantification and statistical analyses

Statistical significance was tested using the two-sided unpaired Student's t-test. N, number of individual experiments, was indicated in each figure legend.

Table 1. Structural properties of the 47 IκBα-interacting and ZnPP-inducible-aggregating proteins.

Gene	Accession	GRAVY[*]	SLIDER[**]	Protein Name
NUP153	P49790	-0.469	0.869	Nuclear pore complex protein Nup153
NUP155	O75694	-0.129	0.701	Nuclear pore complex protein Nup155
IMMT	Q16891	-0.465	0.835	MICOS complex subunit MIC60
EPRS	P07814	-0.517	0.825	Bifunctional glutamate/proline--tRNA ligase
EIF3A	Q14152	-1.489	0.946	Eukaryotic translation initiation factor 3 subunit A
LRPPRC	P42704	-0.206	0.723	Leucine-rich PPR motif-containing protein, mitochondrial
LBR	Q14739	-0.056	0.651	Lamin-B receptor
DDOST	P39656	-0.057	0.417	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase 48 kDa subunit
RANBP2	P49792	-0.596	0.840	E3 SUMO-protein ligase RanBP2
ABCD3	P28288	-0.13	0.518	ATP-binding cassette sub-family D member 3
PDCD6IP	Q8WUM4	-0.468	0.785	Programmed cell death 6-interacting protein
MDN1	Q9NU22	-0.371	0.898	Midasin
RPS2	P15880	-0.233	0.562	40S ribosomal protein S2
MSH6	P52701	-0.499	0.836	DNA mismatch repair protein Msh6
KIF5B	P33176	-0.81	0.876	Kinesin-1 heavy chain
COPB1	P53618	-0.091	0.684	Coatomer subunit beta
USP7	Q93009	-0.643	0.722	Ubiquitin carboxyl-terminal hydrolase 7
RPL10	P27635	-0.553	0.458	60S ribosomal protein L10
HADHA	P40939	-0.078	0.595	Trifunctional enzyme subunit alpha, mitochondrial
DDX6	P26196	-0.323	0.674	Probable ATP-dependent RNA helicase DDX6
TRIM28	Q13263	-0.387	0.857	Transcription intermediary factor 1-beta
COPG1	Q9Y678	-0.145	0.658	Coatomer subunit gamma-1
RPL8	P62917	-0.528	0.539	60S ribosomal protein L8
HACD3	Q9P035	0.003	0.390	Very-long-chain (3R)-3-hydroxyacyl-CoA dehydratase 3
NUP93	Q8N1F7	-0.366	0.705	Nuclear pore complex protein Nup93
HADHB	P55084	-0.072	0.528	Trifunctional enzyme subunit beta, mitochondrial
MCM3	P25205	-0.564	0.879	DNA replication licensing factor MCM3
POLR1C	O15160	-0.28	0.416	DNA-directed RNA polymerases I and III subunit RPAC1
CAND1	Q86VP6	-0.019	0.750	Cullin-associated NEDD8-dissociated protein 1
EIF3M	Q7L2H7	-0.184	0.544	Eukaryotic translation initiation factor 3 subunit M
SLC25A11	Q02978	0.117	0.301	Mitochondrial 2-oxoglutarate/malate carrier protein
BIRC6	Q9NR09	-0.138	0.870	Baculoviral IAP repeat-containing protein 6
PSMA4	P25789	-0.459	0.609	Proteasome subunit alpha type-4
GTF2I	P78347	-0.526	0.798	General transcription factor II-I
IK	Q13123	-1.427	0.928	Protein Red
SLC25A10	Q9UBX3	0.148	0.327	Mitochondrial dicarboxylate carrier
KPNB1	Q14974	-0.092	0.704	Importin subunit beta-1
RPS6	P62753	-0.945	0.820	40S ribosomal protein S6
RPL7A	P62424	-0.547	0.685	60S ribosomal protein L7a
PSMD3	O43242	-0.529	0.821	26S proteasome non-ATPase regulatory subunit 3
SEC16A	O15027	-0.607	0.924	Protein transport protein Sec16A
SEC23A	Q15436	-0.238	0.552	Protein transport protein Sec23A
RPS8	P62241	-1.02	0.651	40S ribosomal protein S8
MCM5	P33992	-0.352	0.671	DNA replication licensing factor MCM5
GTF3C3	Q9Y5Q9	-0.363	0.858	General transcription factor 3C polypeptide 3
IPO4	Q8TEX9	0.07	0.797	Importin-4
XRN2	Q9H0D6	-0.651	0.790	5'-3' exoribonuclease 2
NFKBIA	P25963	-0.452	0.8195	NF-kappa-B inhibitor alpha
NFKBIB	Q15653	-0.388	0.7360	NF-kappa-B inhibitor beta
NFKBIE	O00221	-0.4336	0.8077	NF-kappa-B inhibitor epsilon
SQSTM1	Q13501	-0.632	0.6246	Sequestosome-1

* The GRAVY number of a protein is a measure of its hydrophobicity or hydrophilicity. The hydropathy values range from -2 to +2 for most proteins, with the positively rated proteins being more hydrophobic. Less hydrophobic proteins are labeled red. ** Proteins are listed with SLIDER score, the higher the score the more likely a protein has a long (>= 30 AAs) disordered segment. Scores above 0.538 indicates that a given protein has a long disorder segment (labeled red).

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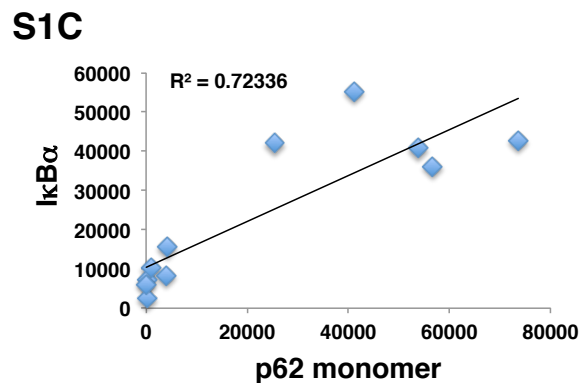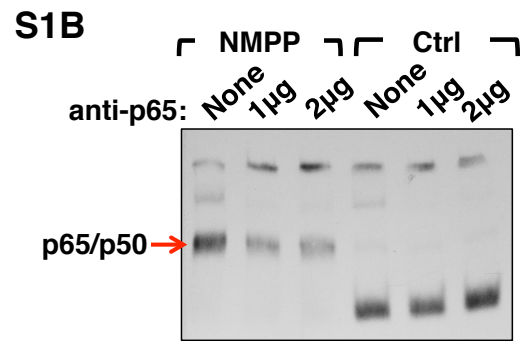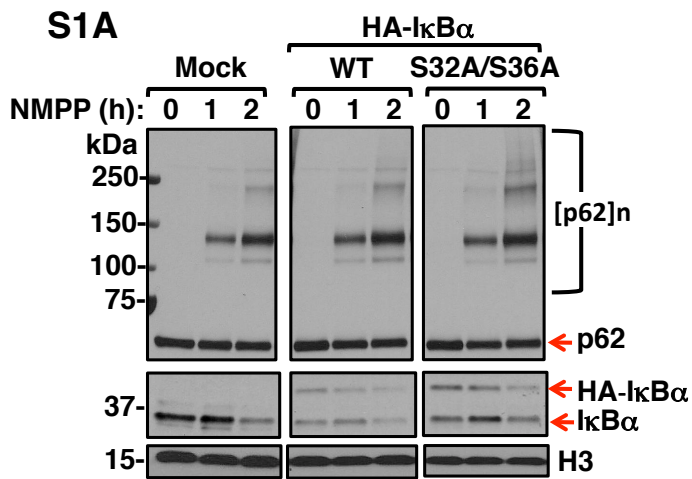
FIGURE S1. NMPP-elicited PPIX-accumulation with concurrent NFκB activation and IκBα-loss is independent of proteasomal degradation, PPIX and ZnPP are even more potent elicitors of IκBα-loss and p62-aggregation than NMPP (Related to Fig 1). **A**. HepG2 cells were transfected with pCMV4-3HA-IκBα or mutant pCMV4-3HA-IκBα-S32A/S36A for 40 h, then treated with NMPP for the indicated times. Cell lysates were subjected to IB analyses of p62 and IκBα with histone H3 as the loading control. **B**. Supershift EMSA using nuclear extracts from Fig 1D with p65-antibody verifies the identity of the p65/p50 band. **C**. The correlation of IκBα and p62 monomer levels quantified from Fig1E (n = 2).
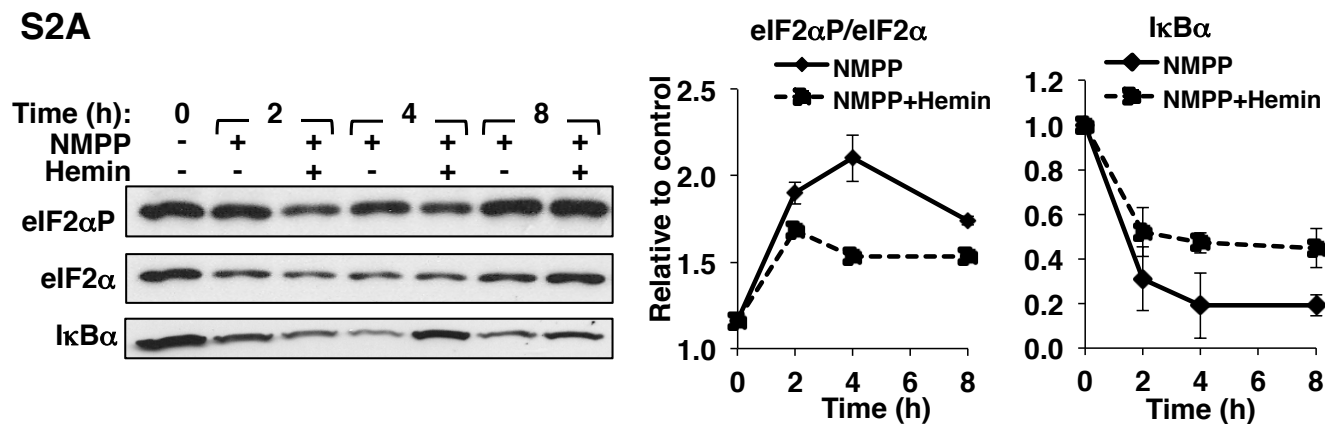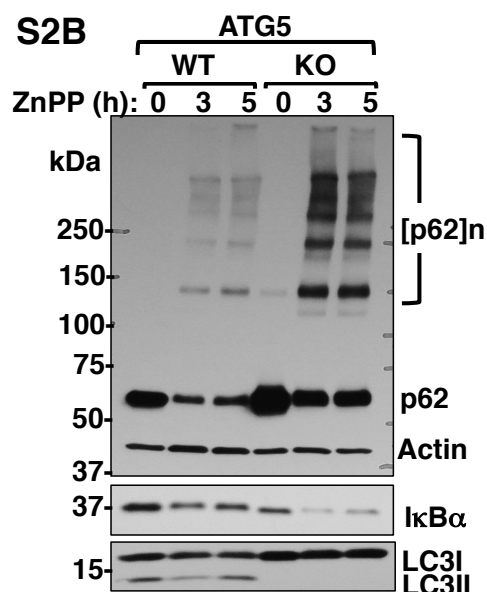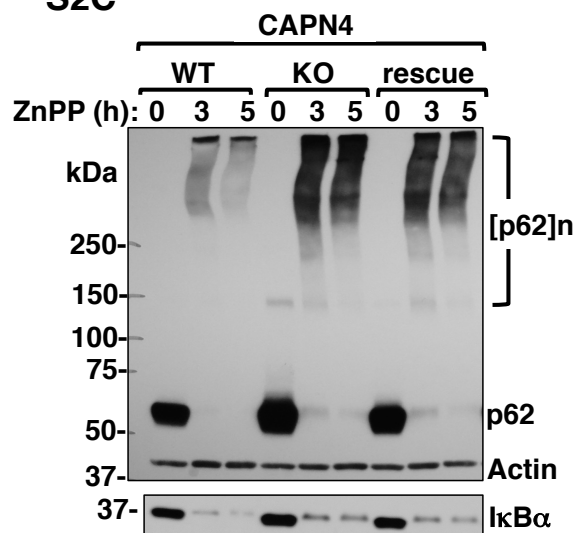
FIGURE S2. ZnPP-elicited IκBα loss is independent of heme deficiency elicited HRI-activation, autophagy and calpain-mediated proteolysis (Related to Fig 1). A. IB analyses of eIF2α, eIF2αP and IκBα in lysates from mouse hepatocytes treated with NMPP or NMPP plus hemin for the indicated times. The temporal profiles of the ratio of eIF2αP to total eIF2α as well as IκBα content were determined (Mean ± SD, n=3). **B**. ATG5 wild-type (ATG5WT) and knockout (ATG5KO) MEF cells were treated with 10 µM ZnPP for the indicated time. Cell lysates were used for IB analysis of p62, IκBα, and LC3, with actin as the loading control. **C**. MEF cells wild-type calpain (capn4WT), capn4 knockout (capn4KO) and capn4KO-rescue (wherein capn4 knockout cells were rescued by transfection of a Capn4 lentiviral vector) were treated with ZnPP for the indicated time. Cell lysates were used for IB analysis of p62 and IκBα with actin as the loading control.
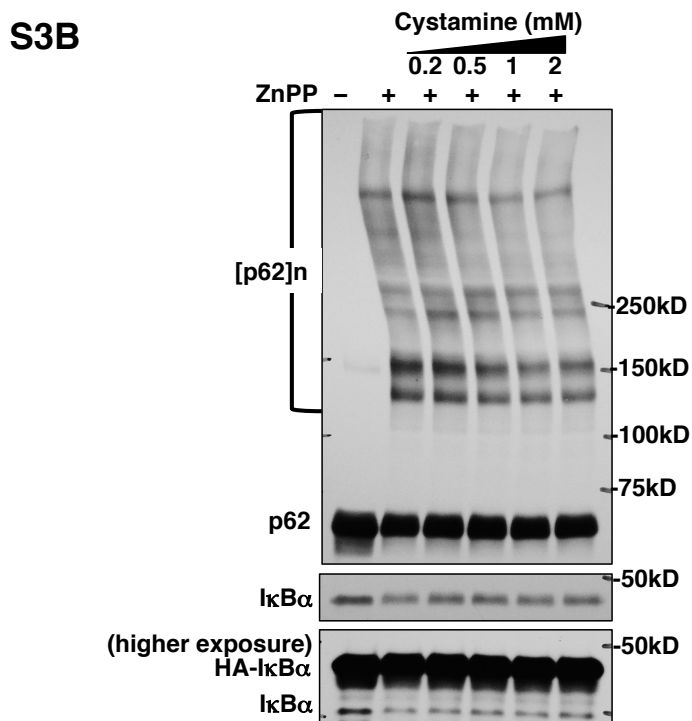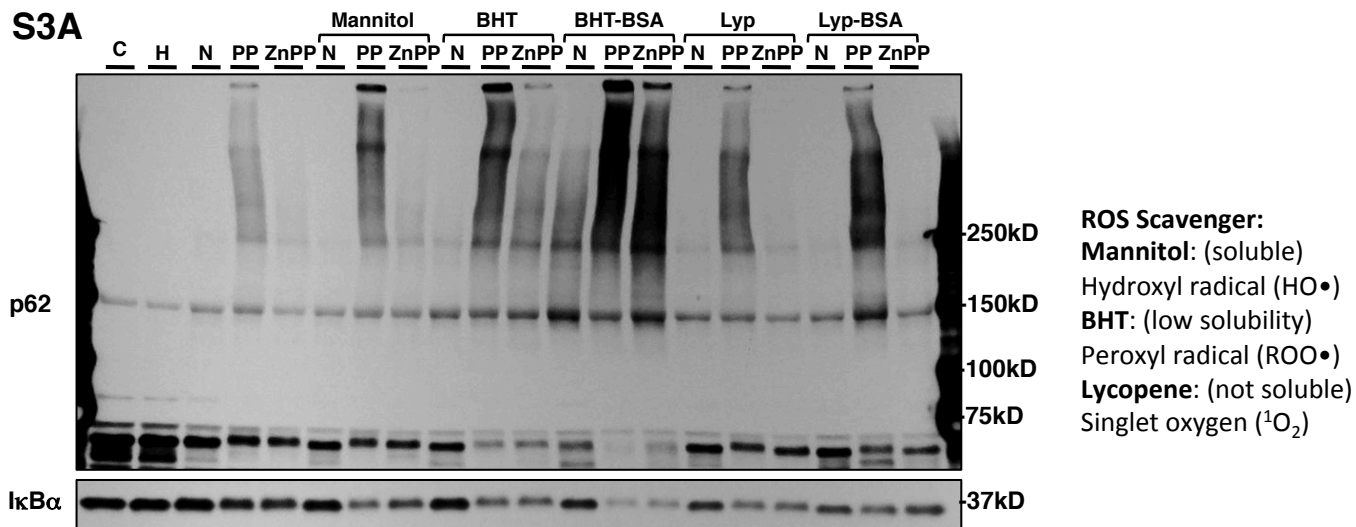
FIGURE S3. ZnPP-elicited IκBα loss and p62 cannot be reversed by antioxidants or TG2 inhibitors (Related to Fig 1). **A**. Primary mouse hepatocytes were left untreated, or pretreated for 1 h with 50mM mannitol, or 100μM BHT, or 50μM lycopene (Lyp) , or BHT coupled with BSA to help keep it solubilized (BHT-BSA), or lycopene coupled with BSA (Lyp-BSA), and then treated with vehicle control (C), 10 μM hemin (H), 30 μM NMPP (N), or 10 μM PPIX (PP), or 10 μM ZnPP (ZnPP) for 4 h as indicated. Cell lysates were used for IB analyses of p62 and IκBα. **B**. HEK293T cells were co-transfected with pcDNA6-p62-myc or pCMV4-3HA-IκBα. 48 h after transfection, cells were pretreated with various concentrations of cystamine as indicated and then treated with 10 μM ZnPP for 2 h. Cell lysates were used for IB analyses of p62 and IκBα.
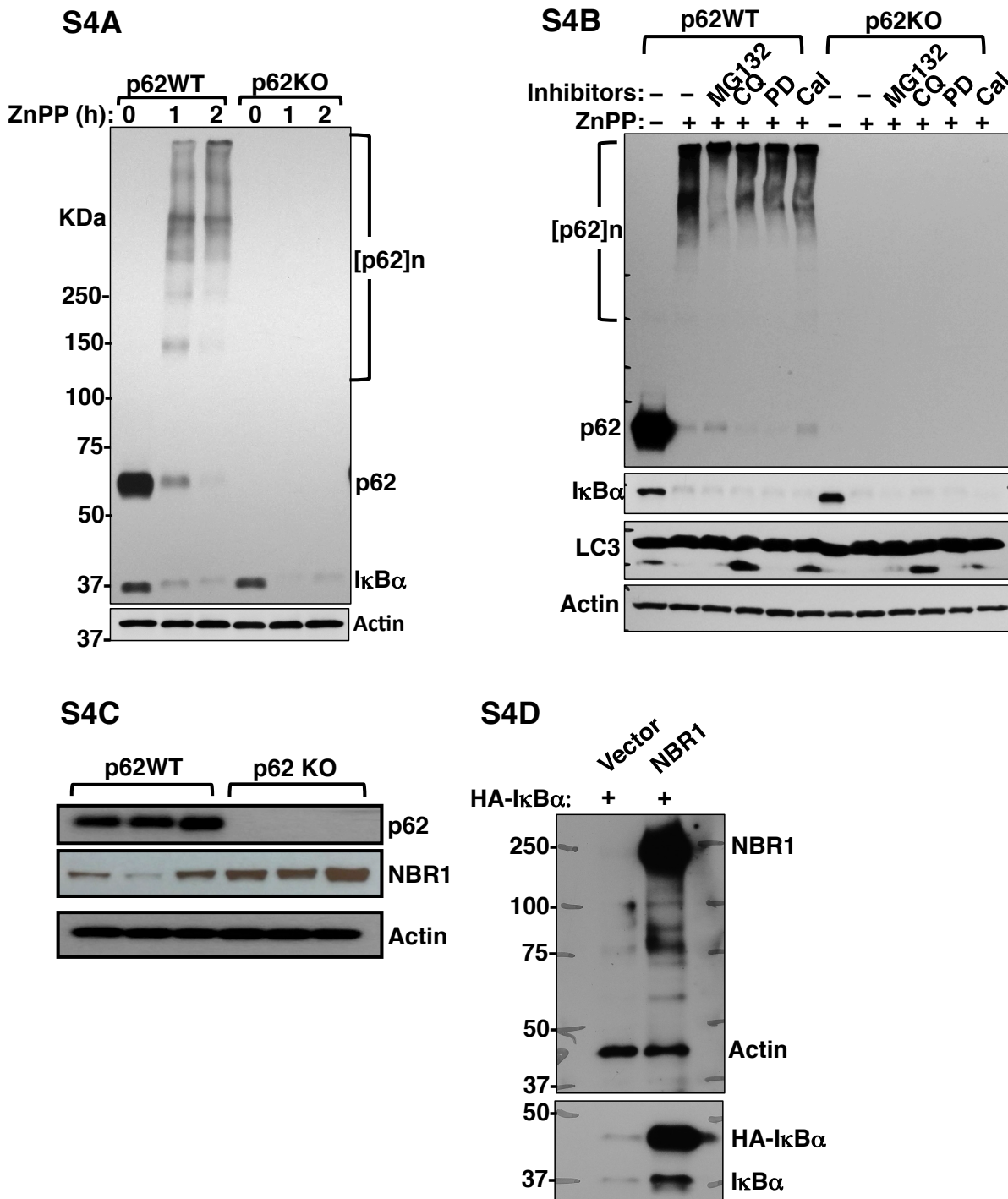
FIGURE S4. ZnPP-elicited IκBα-loss is independent of NBR1 and other autophagic adapters. (Related to Fig 2). A. p62WT and p62KO MEF cells were treated with 10 μM ZnPP for the indicated times. Cell lysates were used for IB analyses of p62 and IkBa, with actin as the loading control. **B.** p62WT and p62KO MEF cells were pretreated for 1 h with inhibitors of various protein degradation pathways: MG132 (20 μM; proteasomal), chloroquine (CQ, 100 μM; lysosomal), PD150606 (PD, 200 μM; calpain1/2), or calpeptin (Cal, 200 μM; cathepsin and calpain), and then treated with 10 μM ZnPP for 2 h. Cell lysates were used for IB analyses. **C.** Lysates from p62WT and p62KO primary mouse hepatocytes were used for IB analyses of p62, and NBR1 with actin as the loading control. The three lanes correspond to p62 WT and p62 KO hepatocytes from three individual mice. **D**. HEK293T cells were co-transfected with pCMV4-3HA-IκBα with either pcDNA3 empty vector or pcDNA3-NBR1 for 48 h. Cell lysates were used for IB analyses of NBR1 and IκBα with actin as the loading control.
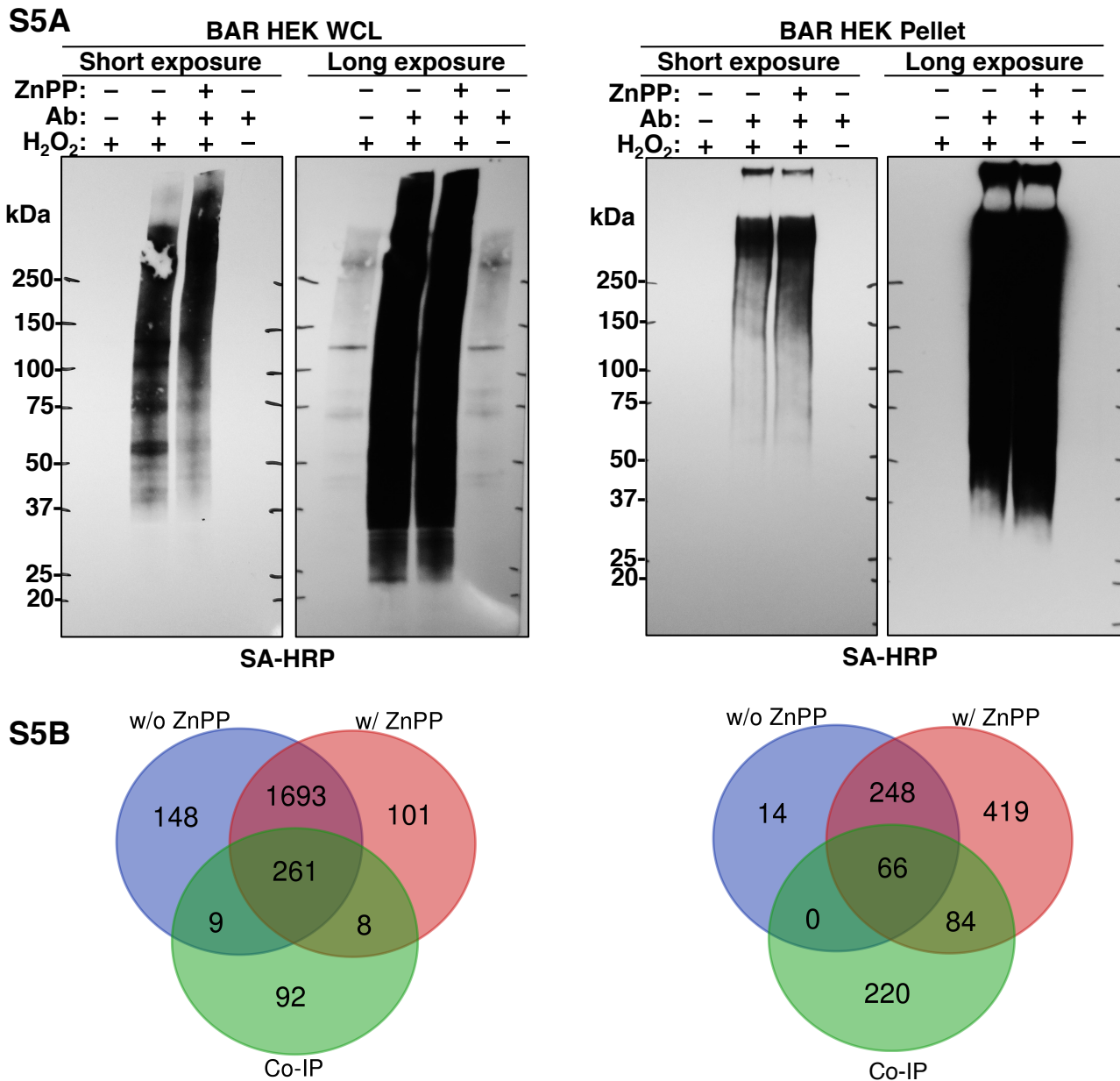
FIGURE S5. Characterization of biotinylation via IκBα antibody recognition (BAR). (Related to Fig 5B). (**A**) HEK293T cells were transfected with with pCMV4-3HA-IκBα for 48 h. Some cells were treated with 10 µM ZnPP for 2 h. Cells were fixed and then stained with IκBα antibody for downstream HRP antibody recognition dependent biotinylation labeling as described in Methods. Cells were lysed, sub-fractionated into whole cell lysates (WCL) and pellet as described in Methods, separated by SDS-PAGE and analyzed by blotting with streptavidin-HRP. Negative controls in which IκBα antibody in substrate recognition or H_2O_2 in labeling reaction was omitted are shown in lanes 1 and 4 of each blot. The band pattern shows that biotinylation is dependent on the presence of both IκBα antibody and H_2O_2, suggesting the specificity of biotinylation of endogenous IκBα-proximal proteins. The up-shift of bands in WCL fraction and an increased intensity of bands in the pellet fraction in ZnPP treated cells suggest that IκBα interacts with ZnPP-aggregated proteins. After higher exposure, in the negative control lanes from WCL fraction, some faint bands representing endogenous biotinylated proteins are detected (i.e.130, 75 and 72 kDa). (**B**). Biotinylated proteins within each lysate (including negative controls) were then enriched using streptavidin-coated magnetic beads and trypsin/ Lys-C digested for LC-MS/MS proteomic analyses. Non-specific protein binders were filtered out by comparison to negative controls, and only proteins with an enrichment ratio of ≥ 4 considered specifically biotinylated by BAR. The numbers of proteins identified using BAR in cells treated with or without ZnPP in comparison with the co-IP method in both fractions are shown in the Venn diagrams.