

1 PHLPP1 Counter-regulates STAT1-mediated Inflammatory Signaling

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15 **ABSTRACT**

16 Inflammation is an essential aspect of innate immunity but also contributes to diverse
17 human diseases. Although much is known about the kinases that control inflammatory signaling,
18 less is known about the opposing phosphatases. Here we report that deletion of the gene
19 encoding PH domain Leucine-rich repeat Protein Phosphatase 1 (PHLPP1) protects mice from
20 lethal lipopolysaccharide (LPS) challenge and live *Escherichia coli* infection. Investigation of
21 PHLPP1 function in macrophages reveals that it controls the magnitude and duration of
22 inflammatory signaling by dephosphorylating the transcription factor STAT1 on Ser727 to
23 inhibit its activity, reduce its promoter residency, and reduce the expression of target genes
24 involved in innate immunity and cytokine signaling. This previously undescribed function of
25 PHLPP1 depends on a bipartite nuclear localization signal in its unique N-terminal extension.
26 Our data support a model in which nuclear PHLPP1 dephosphorylates STAT1 to control the
27 magnitude and duration of inflammatory signaling in macrophages.

28 **HIGHLIGHTS**

- 29 • PHLPP1 controls the transcription of genes involved in inflammatory signaling
- 30 • PHLPP1 dephosphorylates STAT1 on Ser727 to reduce its transcriptional activity
- 31 • PHLPP1 has a nuclear localization signal and a nuclear exclusion signal
- 32 • Loss of PHLPP1 protects mice from sepsis-induced death

33 INTRODUCTION

34 Gene expression is an exquisitely regulated process that maintains cellular homeostasis
35 and orchestrates appropriate responses to environmental stimuli such as hormones, cytokines,
36 and pathogenic microbes (Dawson and Kouzarides, 2012; Flavahan et al., 2017). Homeostatic
37 control of inflammatory genes is particularly relevant to cancer since chronic inflammation
38 promotes tumorigenesis and influences patient response to cancer therapeutics (Coussens and
39 Werb, 2002; Grivennikov et al., 2010). Dysregulated gene expression, a hallmark of cancer, can
40 arise from mutations in transcription factors (exemplified by p53 (Sabapathy and Lane, 2018)),
41 alterations in signaling pathways controlling transcription factor function (for example,
42 hormone-dependent transcription factors in prostate and breast cancers (Jernberg et al., 2017;
43 Pejerrey et al., 2018)), or upregulation of oncogenic transcription factors (notably c-myc, which
44 regulates essential cell-cycle checkpoints (Kalkat et al., 2017)). Aberrant protein phosphorylation
45 underpins all of these mechanisms, via dysregulation of signaling pathways, alterations in
46 transcription factor machinery, and/or effects on the chromatin epigenetic landscape (Rossetto et
47 al., 2012; Whitmarsh and Davis, 2000). Thus, targeting phosphorylation mechanisms is of
48 considerable therapeutic interest.

49 Macrophages are among the first responders to infection, engaging foreign pathogens via
50 pattern recognition receptors, including the Toll-like receptors (TLRs). TLRs are a conserved
51 family of cell surface or phagosome-associated receptors that discriminate distinct features of
52 microbial and viral pathogens, including lipoproteins (TLR1/2/6), lipopolysaccharide (LPS)
53 (TLR4), flagellin (TLR5), single-stranded RNA (TLR7/8), double-stranded RNA (TLR3), and
54 double-stranded DNA (TLR9) (Karin et al., 2006; O'Neill et al., 2013). Upon pathogen
55 recognition by TLRs, a pro-inflammatory response is initiated that activates the signal-dependent

56 transcription factors nuclear factor- κ B (NF κ B), activator protein 1 (AP1), interferon response
57 factors (IRFs), and, through secondary mechanisms, the signal transducer and activator of
58 transcription (STAT) protein family (O'Neill et al., 2013). These activated transcription factors
59 function in a combinatorial manner to drive expression of antimicrobial and inflammatory
60 response genes that aid in elimination of foreign pathogens. However, while inflammation is
61 required for protection against foreign microbes, it can lead to excessive cytokine production,
62 chronic inflammation, and cancer if not properly resolved (Coussens and Werb, 2002; Fullerton
63 and Gilroy, 2016; Grivennikov et al., 2010). Thus, macrophages have evolved regulatory
64 mechanisms to resolve inflammatory responses in a timely manner, including shut down of
65 STAT1 signaling pathways by the suppressor of cytokine signaling (SOCS) family of proteins
66 (O'Shea and Murray, 2008), suppression of nitric oxide production by the enzyme arginase
67 (Wynn and Vannella, 2016), and inhibition of a key subset of NF κ B-dependent genes by anti-
68 inflammatory omega-3 fatty acids, (Oishi et al., 2017).

69 STAT1 is the founding member of the STAT transcription factor family and serves as a
70 paradigm for how phosphorylation regulates transcription factor structure, function, and
71 localization (Darnell et al., 1994; Stark and Darnell, 2012). In the canonical pathway, STATs are
72 recruited from the cytosol to cytokine-bound and Tyr-phosphorylated receptors where they are
73 phosphorylated on a key Tyr residue (Tyr701 for STAT1) by Janus Kinases (JAKs). This
74 phosphorylation event promotes STAT dimerization and nuclear entry, allowing STAT binding
75 to specific promoter sequences and thus initiating gene transcription. Upon promoter binding,
76 STATs become additionally phosphorylated on a regulatory Ser residue at a MAPK consensus
77 sequence (Ser727 for STAT1), a modification that enhances their transcriptional activity
78 (Darnell, 1997; Sadzak et al., 2008; Wen et al., 1995b; Whitmarsh and Davis, 2000).

79 Importantly, STAT1 transduces signals from type I and II interferons (IFNs), resulting in binding
80 to IFN-stimulated response elements (ISREs) and to IFN-gamma (IFN γ)-activated site (GAS)
81 elements in the promoters of IFN-stimulated genes (ISGs), inducing their transcription and
82 stimulating inflammation (Platanias, 2005). While the kinases that phosphorylate Tyr701 and
83 Ser727 on STAT1 have been extensively studied, as have been the phosphatases that
84 dephosphorylate Tyr701, the phosphatases that oppose the Ser727 phosphorylation are unknown.

85 PH domain Leucine-rich Repeat Protein Phosphatase 1 (PHLPP1) is one of the newest
86 members of the phosphatome (Chen et al., 2017; Gao et al., 2005). Originally discovered for its
87 function in suppressing growth factor signaling by dephosphorylating Akt on the hydrophobic
88 motif site, Ser473 (Gao et al., 2005), the repertoire of PHLPP1 substrates is continually
89 expanding (Grzechnik and Newton, 2016). PHLPP1 is a bona fide tumor suppressor: its
90 expression is frequently lost in cancer and its genetic ablation in a mouse model results in
91 prostate neoplasia (Chen et al., 2011; Liu et al., 2009). PHLPP1 is also involved in the immune
92 response, where its dephosphorylation of Akt reduces the capacity of regulatory T cells to
93 transduce T cell receptor signals, a key function in T cell development (Patterson et al., 2011).
94 Recently, PHLPP1 was shown to suppress receptor tyrosine kinase gene expression and
95 influence growth factor signaling, including that mediated by the epidermal growth factor (EGF)
96 receptor (Reyes et al., 2014).

97 PHLPP1 is unusual among protein phosphatases in that its regulatory modules and
98 catalytic domain are on the same polypeptide. Most notably, it has a PH domain essential for
99 dephosphorylation of protein kinase C (PKC) (Gao et al., 2008), a PDZ ligand necessary for Akt
100 recognition (Gao et al., 2005), and a leucine-rich repeat (LRR) segment required for
101 transcriptional regulation of receptor tyrosine kinases (Reyes et al., 2014). In addition, PHLPP1

102 possesses an approximately 50 kDa N-terminal extension (NTE) of unknown function.
103 Stoichiometric association with substrates by direct binding to the protein-interaction domains on
104 PHLPP or common scaffolds (e.g. PDZ domain proteins such as Scribble (Li et al., 2011))
105 allows fidelity and specificity in PHLPP function, and may account for its >10-fold lower
106 catalytic rate compared to the closely related phosphatase PP2C α (Sierecki and Newton, 2014).
107 Given its transcriptional regulation of at least one family of genes, PHLPP1 is an attractive
108 pharmacological target for modulation of gene expression.

109 Here we report that nuclear-localized PHLPP1 opposes STAT1 Ser727 phosphorylation
110 to inhibit its transcriptional activity and promote normal resolution of inflammatory signaling.
111 We find that *Phlpp1*^{-/-} mice have improved survival following infection with *Escherichia coli* (*E.*
112 *coli*), indicating a role of the phosphatase in innate immunity. Since macrophages are key in the
113 initial response to lipopolysaccharide (LPS) from Gram-negative bacteria such as *E. coli*, we
114 further explored the role of PHLPP1 in controlling LPS-dependent signaling in this cell type.
115 The STAT1 binding motif was identified from the most common promoter sequences of 199
116 genes that remained elevated following LPS treatment of bone marrow-derived macrophages
117 (BMDMs) from *Phlpp1*^{-/-} mice compared to those from wild-type (WT) mice. We validated
118 common transcriptional targets of PHLPP1 and STAT1, showing that loss of PHLPP1
119 upregulated the transcription of several genes including guanylate binding protein 5 (*Gbp5*),
120 whereas loss of STAT1 downregulated them. Dephosphorylation of STAT1 on Ser727
121 suppressed its transcriptional activity, and the cellular effects of PHLPP1 depend both on its
122 catalytic activity and a previously undescribed nuclear localization signal (NLS). Taken together,
123 our results identify PHLPP1 as a major player in the resolution of inflammatory signaling.

124 RESULTS

125 PHLPP1 regulates the innate immune response

126 To explore the role of PHLPP1 in acute inflammation, we examined the kinetics and
127 outcome of sepsis-induced death caused by intraperitoneal (i.p.) injection of Gram-negative *E.*
128 *coli* bacteria in WT and *Phlpp1*^{-/-} mice. Surprisingly, absence of PHLPP1 provided a strong
129 protective effect; at a dose where more than 50% of WT mice died within 12 h of *E. coli*
130 challenge, 50% of the *Phlpp1*^{-/-} mice remained alive after 10 days (**Figure 1A**). Similarly,
131 *Phlpp1*^{-/-} mice were protected from toxicity induced by the purified Gram-negative bacterial cell
132 wall component LPS, with nearly half of the *Phlpp1*^{-/-} mice alive after 10 days compared to only
133 1 out of 16 of the WT mice (**Figure 1B**). To understand the lower mortality rates in *Phlpp1*^{-/-}
134 mice, we measured levels of different cytokines in the serum of mice across a time course
135 following LPS injection (**Figure 1C-E**). Serum levels of pro-inflammatory cytokine interleukin 6
136 (IL-6) were significantly increased in WT mice within 5 h of LPS injection, returning to baseline
137 within 12 h (**Figure 1C**). In contrast, the *Phlpp1*^{-/-} mice had 2-fold lower IL-6 levels at 5 h post-
138 infection, but these levels were sustained for up to 24 h, suggestive of improper resolution of
139 inflammation. Levels of another pro-inflammatory cytokine, IL-1 β , were likewise consistently
140 higher in *Phlpp1*^{-/-} mice compared with WT mice (**Figure 1D**). By contrast, levels of anti-
141 inflammatory cytokine IL-10 did not differ significantly between the WT and *Phlpp1*^{-/-} mice
142 (**Figure 1E**). These findings indicate an essential role for PHLPP1 in regulation of the innate
143 immune response at the whole organism level.

144 **Loss of PHLPP1 results in a increased STAT1-dependent transcription in macrophages**

145 Since macrophages are a key cell type involved in the initial response to *E. coli* infection
146 and LPS challenge, we analyzed the transcriptome of BMDMs isolated from WT or *Phlpp1*^{-/-}
147 mice before and after stimulation by the major LPS component, Kdo2-Lipid A (KLA), for 1, 6,
148 or 24 h (**Figure 2A**). RNA-Seq analysis identified 1,654 mRNA transcripts induced more than
149 two-fold by KLA treatment, with a false discovery rate (FDR) less than 0.05 at any of the time
150 points. Expression of approximately 12% of these genes (199 genes; **Table S1**) was increased in
151 macrophages from *Phlpp1*^{-/-} mice compared to those from littermate control WT mice 6 h
152 following KLA treatment; transcript levels of these genes remained significantly elevated (>two-
153 fold) 24 h later. Another set of genes exhibited reduced expression 24 h following KLA
154 treatment (144 genes; **Table S2**). Gene ontology analysis revealed that many of the genes whose
155 expression was elevated in the *Phlpp1*^{-/-} macrophages are associated with inflammatory
156 signaling: these included genes annotated for their involvement in the innate immune response,
157 cytokine-cytokine receptor interactions, LPS signaling, interferon- β response, and tumor
158 necrosis factor (TNF) signaling-dependent pathways (**Figure 2B**). Genes significantly decreased
159 in *Phlpp1*^{-/-} compared to WT macrophages were enriched most significantly in nodes related to
160 central carbon metabolism, and to a lesser extent, chronic inflammatory responses and LPS
161 signaling (**Figure 2B**).

162 To gain insight into gene regulatory mechanisms affected by loss of PHLPP1, we
163 performed *de novo* motif analysis of the promoters of upregulated genes in *Phlpp1*^{-/-}
164 macrophages using Hypergeometric Optimization of Motif EnRichment (HOMER), a suite of
165 tools for motif discovery and Next Generation Sequencing (NGS) analysis (Heinz et al., 2010).
166 This algorithm defines motifs that are statistically enriched in a targeted promoter list compared

167 to random promoter sequences with comparable GC content. The analysis revealed significant
168 enrichment of STAT ($p < 10^{-18}$) and IRF ($p < 10^{-9}$) motifs (**Figure 2C**) in the promoters of genes
169 whose expression was statistically increased in *Phlpp1*^{-/-} macrophages compared to WT
170 macrophages. Of the 199 genes with elevated expression, 46% of the genes had promoters with a
171 consensus STAT binding motif, 51% had promoters with a potential binding site for IRF family
172 of transcription factors, and 26% had promoters with predicted binding sites for both STAT and
173 IRF (**Figure 2D**). We selected for further analysis three genes whose expression was elevated in
174 the *Phlpp1*^{-/-} compared to WT macrophages and which had proximal STAT1 binding motifs in
175 their promoters: *Cd69*, *Ifit2*, and *Gbp5*. Normalized mRNA-Seq data for each of these three
176 genes confirmed elevated mRNA levels in *Phlpp1*^{-/-} macrophages compared to WT macrophages
177 (**Figure 2E-G**). Thus, loss of PHLPP1 leads to sustained KLA-induced expression of genes
178 involved in inflammation, of which 46% have predicted STAT motifs in their proximal
179 regulatory regions.

180 If PHLPP1 suppresses STAT-regulated gene transcription, we reasoned that 1]
181 knockdown of a STAT family member should reduce transcription of the same genes affected by
182 loss of PHLPP1 and 2] knockdown of PHLPP1 should enhance STAT binding to its promoters.
183 STAT1 is required for LPS-induced gene expression in macrophages (Ohmori and Hamilton,
184 2001) and implicated as a PHLPP1 target in iNOS regulation (Alamuru et al., 2014). STAT1
185 knockdown by siRNA in thioglycollate-elicited peritoneal macrophages resulted in a 2-fold
186 reduction in KLA-induced transcription of *Cd69*, *Ifit2*, and *Gbp5* at 6 h compared to a control
187 siRNA transfection, with transcript levels dropping to near baseline by 24 h (**Figure 3A-C**). The
188 effect of PHLPP1 knockdown on STAT1 promoter occupancy was examined by chromatin
189 immunoprecipitation (ChIP) using STAT1-specific antibodies. KLA induced STAT1 binding to

190 the promoters of *Cd69*, *Ifit2*, and *Gbp5*, with maximal binding observed 1 h post stimulation,
191 followed by a decay in binding to near baseline after 24 h (**Figure 3D-F**). In contrast, binding to
192 these promoters was enhanced and sustained in *Phlpp1*^{-/-} macrophages relative to WT cells. The
193 degree of enhancement and the kinetics of activation/resolution varied depending on the gene
194 examined: PHLPP1 loss had the most robust early effect (1 h) on the *Ifit2* promoter and at a later
195 time (24 h) on the *Cd69* promoter. Thus, PHLPP1 suppresses KLA-stimulated binding of STAT1
196 to its promoters and thereby reduces transcription of its target genes.

197

198 **PHLPP1 binds to STAT1 and dephosphorylates Ser727**

199 We next examined whether PHLPP1 affects the phosphorylation state of the two
200 regulatory STAT1 phosphorylation sites, Ser727 and Tyr701. Primary BMDMs were isolated
201 from WT and *Phlpp1*^{-/-} mice and the kinetics and magnitude of KLA-triggered phosphorylation at
202 each of the two STAT1 sites were compared. Loss of PHLPP1 in BMDMs led to a robust
203 increase in STAT1 phosphorylation on the regulatory site Ser727 but did not affect Tyr701
204 phosphorylation (**Figure 4A-B**). PHLPP1 loss also resulted in an increase in Erk
205 phosphorylation at its activation loop sites, as previously reported (Reyes et al., 2014).
206 Incubation of *in vitro* phosphorylated STAT1 with immunoprecipitated FLAG-tagged PHLPP1
207 resulted in dephosphorylation at Ser727, suggesting that PHLPP1 directly dephosphorylates
208 STAT1 (**Figure 4C**). Furthermore, overexpression of PHLPP1 in HEK-293T cells reduced
209 IFN γ -dependent phosphorylation of STAT1 on Ser727 but not on Tyr701 (**Figure 4D-E**). Thus,
210 PHLPP1 selectively dephosphorylates the Ser727 regulatory phosphorylation on STAT1 in cells
211 and *in vitro*.

212 Because the abundance of PHLPP1 in the cell is much lower than other phosphatases
213 such as PP2A (Hein et al., 2015), we next sought to determine whether regulation of STAT1
214 promoter activity was solely due to PHLPP1 phosphatase activity or occurred in combination
215 with other phosphatases. Taking advantage of the insensitivity of PHLPP phosphatases to the
216 PP1/PP2A inhibitor okadaic acid (OA) (Gao et al., 2005), we examined whether OA treatment
217 affected KLA-dependent changes on Ser727 phosphorylation in primary BMDMs from WT
218 mice. **Figure 5A-B** shows that the KLA-induced increase in Ser727 phosphorylation was
219 relatively insensitive to OA, under conditions where the phosphorylation of Erk (at
220 Thr202/Tyr204) and Akt (at Thr308) was significantly increased upon OA addition. These data
221 are consistent with PHLPP1, a PP2C family member, being the primary regulator of
222 phosphorylation on the activity-tuning Ser727 site of STAT1.

223 We next addressed whether enhanced promoter binding of STAT1 upon loss of PHLPP1
224 resulted in enhanced transcriptional activation using a luciferase reporter assay. WT or *Phlpp1*^{-/-}
225 mouse embryonic fibroblasts (MEFs) were co-transfected with a firefly luciferase reporter
226 construct containing GAS promoter elements, as well as a renilla luciferase controlled by a
227 constitutive CMV promoter as an internal control. STAT1 promoter activity was assessed by
228 monitoring luminescence following IFN γ stimulation. **Figure 6A** shows that STAT1 promoter
229 activity was significantly higher in *Phlpp1*^{-/-} MEFs compared to WT MEFs at both 6 h and 24 h.
230 Pre-treatment of cells with okadaic acid, under conditions that increased the phosphorylation of
231 PP2A-sensitive substrates (see **Figure 5A**), had no effect on STAT1 promoter activity (**Figure**
232 **6A**). Because STAT1 functions in the nucleus, we next asked whether PHLPP1 regulation of
233 STAT1 occurs in the cytoplasm or nucleus. To this end, we assessed the effect of expressing
234 either the PP2C domain of PHLPP1 or a nuclear-targeted (NLS) PP2C domain of PHLPP1 on

235 IFN γ -induced STAT1 promoter activity via the GAS luciferase assay (**Figure 6B**). The
236 overexpressed PP2C domain of PHLPP1 (**Figure 6C, blue**) was considerably less effective in
237 inhibiting STAT1 promoter activity compared to full-length PHLPP1 (**Figure 6B, red**).
238 However, forcing the PP2C domain into the nucleus by attaching an NLS to its N-terminus
239 inhibited STAT1 promoter activity as effectively as overexpression of full-length PHLPP1
240 (**Figure 6B, orange**). Analysis of the subcellular localization of the constructs used in this
241 experiment revealed that full-length PHLPP1 was primarily cytosolic, the isolated PP2C domain
242 had increased nuclear localization, and the NLS-PP2C was enriched in the nucleus (**Figure S2**).
243 To address whether PHLPP1 catalytic activity is required for STAT1 regulation, we utilized a
244 phosphatase-dead PP2C domain in which two active site residues, Asp1210 and Asp1413
245 (Sierecki and Newton, 2014) were mutated to Ala (DDAA). The catalytically-inactive NLS-
246 PP2C was no longer able to suppress STAT1 activity (**Figure 6C, purple**);
247 immunohistochemistry confirmed its nuclear localization (**Figure S1**). Thus, both the catalytic
248 activity and nuclear localization of PHLPP1 are necessary for it to regulate STAT1
249 transcriptional activity.

250

251 **PHLPP1 has a bipartite Nuclear Localization Signal in its N-Terminal Extension**

252 Bioinformatics analysis of the sequence of PHLPP1 using SeqNLS (Lin and Hu, 2013)
253 revealed a potential Arg-rich bipartite NLS (⁹²RRRRR-X-¹²²RRGRLKR) in the N-terminal
254 extension unique to the PHLPP1 isozyme (**Figure 6D**). To test whether these basic segments
255 function as an NLS, we examined the subcellular localization in HeLa cells of the NTE alone or
256 NTE in which the basic residues in each or both halves of the potential bipartite NLS were
257 mutated to Ala (**Figure 6E**). Immunocytochemistry revealed that the NTE localizes to the

258 nucleus. Mutation of the first NLS or the second NLS increased the distribution of the NTE to
259 the cytosol, and mutation of both decreased the nuclear:cytoplasmic ratio to be comparable to
260 that of a construct of the NTE with a strong Nuclear Exclusion Signal (NES) (LALKLAGLDI
261 from PKI (Wen et al., 1995a) (**Figure 6F**). Full-length PHLPP1 was primarily cytosolic, leading
262 us to ask whether there may also be an NES. Bioinformatics analysis of the primary sequence
263 identified a potential Leu-rich NES (Fu et al., 2011) immediately following the last LRR and
264 preceding the phosphatase domain (**Figure S3**). Attachment of this 14-residue sequence to the N-
265 terminus of the NTE resulted in distribution of the NTE to the cytosol (**Figure S3**). Thus,
266 PHLPP1 nuclear localization is controlled by a bipartite NLS in the NTE and is opposed by an
267 NES following the LRR. Lastly, we examined the effect of mutating the NLS on the ability of
268 full-length PHLPP1 to reduce STAT1 transcriptional activity as assessed using the GAS
269 promoter assay. The reduction in IFN γ -induced STAT1 activity resulting from PHLPP1
270 overexpression (**Figure 6G**, red) was abolished upon mutation of NLS2 (**Figure 6G**, brown) or
271 both halves of the NLS (NLS1/2) (**Figure 6G**, purple). Mutation of NLS1 had an intermediate
272 effect (**Figure 6G**, blue). These data reveal that a bipartite NLS in the NTE of PHLPP1 localizes
273 PHLPP1 to the nucleus, where it suppresses the transcriptional activity of STAT1.

274 We next assessed which domain of PHLPP1 contributes to the observed regulation of
275 STAT1 activity on the GAS promoter. Overexpression of full-length PHLPP1 in HEK-293T
276 cells markedly reduced GAS promoter activity (**Figure 7A**, red) compared to the vector only
277 control (**Figure 7A**, black). A construct of PHLPP1 lacking the NTE (deletion of first 512 amino
278 acids of its N-terminus; P1 Δ NTE, blue) was less effective than full-length PHLPP1 in reducing
279 STAT1 activity, whereas a construct comprised of just the NTE (amino acids 1-512, green)
280 caused a significant increase in GAS promoter activity, suggesting a dominant-negative function

281 of this segment. Co-immunoprecipitation assays revealed a robust interaction of STAT1 with the
282 immunoprecipitated NTE of PHLPP1, in contrast to barely detectable binding of STAT1 to
283 PHLPP1 lacking the NTE (**Figure 7B**). Intermediate binding was observed between STAT1 and
284 full-length PHLPP1. Quantification of three independent experiments revealed that the isolated
285 NTE of PHLPP1 binds STAT1 approximately five times more strongly than full-length PHLPP1
286 and 26 times more strongly than PHLPP1 lacking the NTE (**Figure 7C**). These data reveal that
287 the NTE of PHLPP1 interacts with STAT1 and reduces its promoter activity.

288

289

290 **DISCUSSION**

291 The finding that *Phlpp1*^{-/-} mice are protected from LPS-induced death allowed us to identify
292 PHLPP1 as a physiologically relevant phosphatase in the overall innate immune response. It is
293 likely that this immunoregulatory phenotype reflects roles of PHLPP1 in several immune cell
294 types, and future studies of mice with cell-specific deletions of *Phlpp1* will be of great interest.
295 Investigation of *Phlpp1*^{-/-} macrophages indicates a significant role in counter-regulation of
296 STAT1-dependent transcription that emerges as a secondary response to TLR4 ligation. Our
297 mechanistic analyses show that PHLPP1 dephosphorylates STAT1 on a key regulatory site to
298 suppress its transcriptional activity towards an array of genes involved in mounting an
299 inflammatory response to IFN γ . Specifically, PHLPP1 directly dephosphorylates Ser727 on
300 STAT1 *in vitro* and specifically suppresses phosphorylation of Ser727, but not Tyr701, on
301 STAT1 in cells, correlating to decreased transcriptional activity of STAT1 at one of its major
302 binding sites, the GAS promoter. The intrinsic catalytic activity and nuclear localization of
303 PHLPP1 is required for this transcriptional regulation; while the isolated PP2C domain is not
304 efficient in suppressing GAS promoter activity, forcing the PP2C domain into the nucleus is as
305 effective as the full-length phosphatase in controlling transcriptional activity. Nuclear
306 localization of the full-length enzyme is driven by a bipartite NLS we identify in the NTE.
307 Elimination of PHLPP1 results in global changes in LPS-dependent transcriptional regulation,
308 with 20% of the approximately 2,000 genes whose expression changes upon LPS stimulation
309 differing by more than two-fold in BMDMs from *Phlpp1*^{-/-} mice compared to WT mice.

310 Phosphorylation of STAT1 on Ser727 has been proposed to occur following the binding of
311 the Tyr-phosphorylated STAT1 dimer to chromatin (Sadzak et al., 2008). Ser727
312 phosphorylation on the C-terminal transactivation domain of STAT1 is necessary for maximal

313 transcriptional activity. Identification of PHLPP1 as a phosphatase that opposes this
314 phosphorylation provides a mechanism to counter-regulate the activity of this key transcription
315 factor. Several lines of evidence suggest that PHLPP1 may be the major phosphatase that
316 controls this regulatory site. First, genetic depletion of PHLPP1 increases both STAT1 Ser727
317 phosphorylation and transcriptional activity at the GAS promoter, whereas PHLPP1
318 overexpression decreases both STAT1 Ser727 phosphorylation and transcriptional activity at the
319 promoter. Second, both the IFN γ -induced phosphorylation of Ser727 and resulting increase in
320 transcriptional activity at the GAS promoter are insensitive to OA, a phosphatase inhibitor that is
321 ineffective towards PP2C family members but highly effective towards the abundant PP2A in
322 cells. The insensitivity of STAT1 Ser727 phosphorylation to OA is consistent with PHLPP1
323 directly dephosphorylating this site in cells, a reaction it catalyzes *in vitro*. Furthermore, although
324 PHLPP1 does suppress the signaling output of Akt (by dephosphorylating Ser473 (Gao et al.,
325 2005)) and Erk (by reducing the steady-state levels of RTKs (Reyes et al., 2014)), its effect on
326 STAT1 is unlikely to involve either of these targets because the activities of both kinases are
327 sensitive to OA. Nor are the effects on Ser727 a result of PHLPP1 reducing PKC steady-state
328 levels (Baffi et al., 2019), as the general PKC inhibitor Gö6983 did not alter GAS promoter
329 activity (**Figure S4**). Third, genetic depletion of either PHLPP1 or STAT1 has opposing effects
330 on transcriptional targets of STAT1: whereas KLA causes a larger increase in mRNA of *Cd69*,
331 *Ifit2*, and *Gbp5* in BMDMs from *Phlpp1*^{-/-} mice compared to WT mice, a reduction in these
332 transcripts is observed upon STAT1 knockdown. Lastly, we have previously shown that PHLPP1
333 regulates transcription of genes and binds chromatin (Reyes et al., 2014). Cumulatively, these
334 data are consistent with PHLPP1 being the major phosphatase to oppose the activating
335 phosphorylation of STAT1 on Ser727, thereby limiting its transcriptional activity.

336 The interaction of PHLPP1 with STAT1, mediated by its NTE, affords fidelity and
337 specificity in its dephosphorylation of the transcription factor. PHLPP1 binding to STAT1 is
338 consistent with this multi-valent protein utilizing its protein-interaction domains to position it
339 near its substrates, either via direct interaction or by binding protein scaffolds, such as PDZ
340 domain proteins that coordinate Akt signaling (Li et al., 2011). Such coordination is essential for
341 its dephosphorylation of relevant substrates, in part due to the low catalytic activity of the
342 phosphatase (approximately 1 reaction per sec towards peptide substrates, an order of magnitude
343 lower than that of the related phosphatase PP2C α (Sierecki and Newton, 2014)). The importance
344 of enzyme proximity to its substrate is best illustrated with Akt, where deletion of the last 3
345 amino acids of PHLPP1 to remove the PDZ ligand abolishes the ability of PHLPP1 to
346 dephosphorylate Akt in cells (Gao et al., 2005). Thus, binding of PHLPP1 via its NTE to STAT1
347 affords an efficient mechanism to restrict its activity by directly opposing its phosphorylation in
348 the nucleus (see **Figure 8**).

349 The regulation of STAT1 by PHLPP1 occurs in the nucleus, and we identify motifs in the
350 phosphatase that control both the entry into (NLS) and exit from (NES) the nucleus. First, we
351 identify a bipartite NLS in the NTE of PHLPP1 whose integrity is necessary for the phosphatase
352 to regulate the transcriptional activity of STAT1. Second, we identify an NES in the segment
353 between the LRR and PP2C domain that drives export out of the nucleus. Under the
354 ‘unstimulated’ conditions of our immunocytochemistry, PHLPP1 localized primarily to the
355 cytosol, suggesting masking of the NLS and exposure of the NES. Inputs that regulate the
356 exposure of the NLS and NES are likely important regulators of PHLPP1 function.

357 Our transcriptomic data support a key role for PHLPP1 in the resolution of the
358 inflammatory response specific to genes downstream of type II IFN signaling pathways. This

359 suggests the possibility that PHLPP1 can selectively discriminate between inflammatory
360 promoters that are differentially regulated by distinct transcription factor families. Surprisingly,
361 over 50% of the inflammatory genes that fail to properly resolve in the macrophages from
362 *Phlpp1*^{-/-} mice contain a consensus STAT-binding motif in their proximal promoters. Our studies
363 have demonstrated a direct interaction between PHLPP1 and STAT1, thus it is highly likely that
364 PHLPP1 is recruited to gene promoters through its association with STAT1. Elevated STAT1
365 occupancy and delayed dismissal kinetics of STAT1 from its target promoters in *Phlpp1*^{-/-}
366 macrophages indicate a major function of PHLPP1-dependent dephosphorylation in termination
367 of STAT1 signaling and its dismissal from chromatin.

368 Germline mutations that impair STAT1 function, by reducing either Tyr701 phosphorylation
369 (L706S) or DNA binding (Q463H and E320Q), increase the susceptibility of otherwise healthy
370 patients to mycobacterial and viral infection (Chapgier et al., 2006; Dupuis et al., 2001). This
371 increased susceptibility was proposed to arise because of reduced transcription of genes involved
372 in bacterial and viral immunity from the GAS and ISRE promoters, respectively. Similarly,
373 genetic ablation of *STAT1* on the background of a mouse that has enhanced TLR4 signaling
374 (because of deletion of *gp130*, a key regulator of systemic inflammatory responses during LPS-
375 mediated endotoxemia) provides protection against LPS-induced toxemic death compared to
376 mice with normal STAT1 levels (Luu et al., 2014). Given the protective effect of PHLPP1 loss
377 on both *E. coli*-induced sepsis and LPS-induced endotoxemia in mice, it is possible that PHLPP1
378 inhibitors could be explored as adjunctive therapies to antibiotics and supportive care of patients
379 with Gram-negative sepsis, a leading cause of mortality in intensive care units.

380 **EXPERIMENTAL PROCEDURES**

381 **Materials and Antibodies**

382 OA (459616) was purchased from Millipore. Gö6983 (365251) and staurosporine (569397) were
383 purchased from Calbiochem. Antibody against HA (11867425001) was purchased from Roche;
384 antibodies against GFP (2555), STAT1 (9172), phosphorylated Ser727 on STAT1 (9177),
385 phosphorylated Tyr701 on STAT1 (7649), phosphorylated Thr202/Tyr204 on Erk1/2 (9101),
386 total Erk1/2 (9102), and phosphorylated Thr308 on Akt (9275) were purchased from Cell
387 Signaling. Antibody against total Akt (126811) was obtained from AbCam. Antibodies against
388 PHLPP1 were purchased from Cosmo (KIAA0606) and Proteintech (22789-1-AP); antibodies
389 against FLAG (F3165), β -Actin (A2228), and α -tubulin (T6074) were purchased from Sigma-
390 Aldrich. The pcDNA3 HA-tagged PHLPP1 and PHLPP2 constructs for mammalian cell
391 expression were described previously (Brognard et al., 2007; Gao et al., 2008; Gao et al., 2005).
392 Full-length PHLPP1 was cloned into pCMV 3xFLAG vector (Sigma-Aldrich, E4401). An NLS
393 was cloned to the N-terminus of the PP2C domain of PHLPP1. A double mutant of NLS-PP2C at
394 residues D1210A and D1413A was cloned by site-directed mutagenesis. The HA-tagged
395 PHLPP1 N-terminal extension (PHLPP1 NTE), residues 1-512, was cloned into pcDNA3 vector
396 (Invitrogen). The NLS1 and NLS2 mutations were cloned by site-directed mutagenesis into HA-
397 PHLPP1 NTE. The NES from PKI (LALKLALDI) was cloned into the N-terminus of HA-
398 PHLPP1 NTE. The PHLPP1 NES mutant was generated by site-directed mutagenesis.

399 **Isolation and treatment of macrophages**

400 Primary BMDM cells were isolated from male 6- to 8-week-old C57BL/6 mice (Charles River
401 Laboratories). BMDMs were obtained by PBS flush of femurs and tibias (Weischenfeldt and
402 Porse, 2008), red blood cells lysed, and remaining cells plated in RPMI 1640 supplemented with

403 20% fetal bovine serum (FBS, Gibco, cat. 12657-029), 30% L-cell conditioned medium, 100
404 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Cells were seeded in non-
405 tissue culture treated Optilux Petri dishes (BD Biosciences), incubated at 37 °C in a 5% CO₂
406 atmosphere for 7 days, then treated with 100 ng/ml KLA (699500, Avanti Polar Lipids) for noted
407 times. Peritoneal macrophages were collected by flushing mouse peritoneal cavities with PBS
408 following 48 hours post peritoneal injection with 3 ml of thioglycolate (Ray and Dittel, 2010).

409 **Cell culture**

410 MEFs from WT or *Phlpp1*^{-/-} mice stably expressing shp53 were a kind gift from Lloyd Trotman
411 (CSHL) and have been described previously (Chen et al., 2011); MEFs, HEK-293T, and HeLa
412 cells were grown in Dulbecco's modified Eagle medium (DMEM, 10-013-CV, Corning)
413 supplemented with 10% fetal bovine serum (S11150, Atlanta biologicals) and 1%
414 penicillin/streptomycin (15140-122, Gibco) at 37 °C in 5% (vol/vol) CO₂.

415 **mRNA isolation and qPCR analysis:**

416 RNA was purified using Direct-zol RNA Miniprep Kits (Zymo Research) from triplicate
417 experiments and quantified using a NanoDrop Spectrophotometer (ThermoFisher Scientific).
418 RNA was either reverse transcribed into cDNA for quantitative real-time PCR using gene-
419 specific primers or used for next-generation library preparation. For cDNA generation, one µg of
420 total mRNA was reverse transcribed using the SuperScript III Reverse Transcriptase
421 (ThermoFisher Scientific). The resulting cDNA (25 ng) was used to perform real-time PCR
422 using SYBR Green Master Mix (ThermoFisher Scientific) and 50 nM mix of forward and
423 reverse primers. The real-time PCR values for individual genes were normalized to the house
424 keeping gene, 36B4, using the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001). The primer
425 sequences used in this study are:

426 *36B4_qPCR_F* AATCTCCAGAGGCACCATTG
427 *36B4_qPCR_R* CCGATCTGCAGACACAACT
Cd69_qPCR_F CTATCCCTTGGGCTGTGTTAAT
Cd69_qPCR_R ACATGGTGGTCAGATGATTCC
Ifit2_qPCR_F GAGTTTGAGGACAGGGTGTTTA
Ifit2_qPCR_R AGACCTCTGCAGTGCTTTAC
Gbp5_qPCR_F GGAAGTGCTGCAGACCTATT
Gbp5_qPCR_R GCTCTTTCTTGTTCCGCTTTAC

428 **Next-generation sequence library preparation and analysis**

429 Libraries were prepared from 2 biological replicates per condition. RNA-Seq libraries were
430 prepared as previously described (Kaikkonen et al., 2013). Sequencing libraries were prepared
431 using magnetic beads similar to described previously using barcoded adapters (NextFlex, Bioo
432 Scientific) (Garber et al., 2012). Libraries were sequenced for 36 or 50 cycles on an Illumina
433 Genome Analyzer II or HiSeq 2000, respectively, according to the manufacturer's instructions.
434 mRNA-Seq results were trimmed to remove A-stretches originating from the library preparation.
435 Each sequence tag returned by the Illumina Pipeline was aligned to the mm10 assembly using
436 ELAND allowing up to 2 mismatches. Only tags that mapped uniquely to the genome were
437 considered for further analysis. Peak finding, MOTIF discovery, and downstream analysis was
438 performed using HOMER, a software suite created for analysis of high-throughput sequencing
439 data (Heinz et al., 2010). Detailed instructions for analysis can be found at
440 <http://homer.ucsd.edu/homer/>. Data visualization was performed using Microsoft Excel,
441 JavaTreeGraph and software packages available in R.

442 **RNA interference experiments**

443 SMART siRNA pools for examined genes were purchased from Dharmacon (Control: D-
444 001810-10-05, *Stat1*: L-058881). Thioglycollate-elicited peritoneal macrophages were
445 transfected with 30 nM siRNA for 48 h using Deliver X (Affymetrix) according to the
446 manufacturer's instructions prior to being stimulated with KLA for designated times.

447 **Chromatin immunoprecipitation**

448 ChIP assays were performed as described before (Stender et al., 2017). Cells were crosslinked
449 with 2 mM disuccinimidyl glutarate for 30 min prior to 10 min treatment with 1% formaldehyde.
450 The antibodies used in these studies were: STAT1 (sc-345, Santa Cruz Biotechnology). For the
451 precipitations protein A Dynabeads (10003D, Invitrogen) were coated with antibody prior to
452 pulldown and excess antibody was washed away. Pulldowns occurred while rotating for 16 h at 4
453 °C. Beads were then washed with TSE I (20 mM Tris/HCl pH 7.4 at 20 °C, 150 mM NaCl, 0.1%
454 SDS, 1% Triton X-100, 2 mM EDTA), twice with TSE III (10 mM Tris/HCl pH 7.4 at 20 °C,
455 250 mM LiCl, 1% IGEPAL CA-630, 0.7% Deoxycholate, 1 mM EDTA), and twice with TE
456 followed by elution from the beads using elution buffer (0.1 M NaHCO₃, 1% SDS). Elutions
457 were subsequently de-crosslinked overnight at 65 °C and DNA was purified using ChIP DNA
458 Clean and Concentrator (Zymo Research) and DNA was used for qPCR. The primer sequences
459 used in this study are:

*Cd69*_ChIP_F TCCCTGCTGTCTGAAATGTG

*Cd69*_ChIP_R GTGGAAGGATGTCTTCGATTCT

*Ifit2*_ChIP_F GCATTGTGCAAGGAGAATTCTATG

*Ifit2*_ChIP_R TTCCGGAATTGGGAGAGAGA

*Gbp5*_ChIP_F TAAACAGCGCTTGAAACAATGA

*Gbp5*_ChIP_R AGGCTTGAATGTCACTGAACTA

460 **Luciferase assay**

461 Cells were plated in a 96-well plate and transfected when approximately 80% confluent.
462 Transfections of pRL-CMV encoding Renilla luciferase (Heinz et al., 2010), together with a
463 firefly luciferase promoter-reporter construct containing eight GAS consensus sequences (Horvai
464 et al., 1997), control vector, or the indicated PHLPP constructs, were performed using
465 Lipofectamine 3000 reagent (Invitrogen, L3000) for MEFs or Fugene 6 reagent (Promega,
466 E269A) for HEK-293T cells. Cells were treated with murine or human IFN γ (PeproTech, 315-
467 05, 300-02, respectively) for the indicated times at 37 °C and activity was measured using the
468 Dual-Glo Luciferase Assay System (Promega, E2940) in a Tecan Infinite M200 Pro multi-well
469 plate reader. Promoter activity was corrected for the luciferase activity of the internal control
470 plasmid, pRL-CMV, and Relative Response Ratios (RRR) were calculated.

471 **Immunoprecipitation and western blot**

472 DNA was transfected into HEK-293T cells using FuGene 6. Cells were collected 24 h post-
473 transfection and then lysed in a buffer containing 50 mM Na₂HPO₄ (pH 7.5), 1 mM sodium
474 pyrophosphate, 20 mM NaF, 2 mM EDTA, 2 mM EGTA, 1% SDS, 1 mM DTT, 1 μ M
475 microcystin, 20 μ M benzamidine, 40 μ g/ml leupeptin, and 1 mM PMSF and then were sonicated
476 briefly. For co-immunoprecipitation, cells were lysed and the detergent-solubilized cell lysates
477 were incubated with an anti-HA antibody (BioLegend, 901503) at 4 °C overnight. Samples were
478 incubated with protein A/G PLUS-Agarose (Santa Cruz Cat sc-2003) for 1 h at 4 °C and washed
479 three times in lysis buffer containing 0.3 M NaCl and 0.1% Triton X 100. Bound proteins and
480 lysates were separated by SDS/PAGE gel and analyzed by western blot.

481 **Immunofluorescence**

482 HeLa cells were plated on glass coverslips and transfected using FuGene 6. 24 h after
483 transfection, cells were fixed with 4% paraformaldehyde for 20 min at room temperature,
484 followed by fixation with 100% methanol for 3 min at -20 °C. Cells were permeabilized and
485 blocked in 0.3% Triton X 100 and 3% BSA for 30 min at room temperature, followed by three 5-
486 min washes in PBS-T. Primary antibodies were diluted at the following dilutions: mouse anti-
487 HA, 1:500; rabbit anti- α -tubulin (Cell Signaling, 2125), 1:200. Secondary antibodies were
488 diluted at the following dilutions: Alexa647 anti-mouse (Life Technologies, A21235), 1:500;
489 Alexa488 anti-rabbit (Life Technologies, A11034), 1:500. Coverslips were mounted onto slides
490 with ProLong Diamond Antifade Mountant with DAPI (ThermoFisher, P36966). Images were
491 acquired on a Zeiss Axiovert 200M microscope (Carl Zeiss Microimaging Inc.) using an iXon
492 Ultra 888 EMCCD camera (ANDOR) controlled by MetaFluor software (Molecular Devices)
493 and analyzed on ImageJ (NIH). The Nuclear to Cytoplasmic ratio was calculated as follows: the
494 mean signal intensity was measured for a region of the nucleus and cytoplasm for each cell, and
495 the mean signal intensity of the background was subtracted from these values. Then the Nuclear
496 to Cytoplasmic ratio was calculated by dividing the background subtracted mean signal intensity
497 for the nuclear signal by the background subtracted value for the cytoplasmic signal.

498 ***In vitro* phosphatase assay**

499 pCMV 3xFLAG PHLPP1 was transfected into HEK-293T cells plated in four 10 cm plates
500 (approximately 9×10^6 cells per plate, 80% transfection efficiency) using Fugene 6. Cells were
501 collected 48 h post-transfection and lysed in a buffer containing 20 mM Tris (pH 7.5), 150 mM
502 NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X 100, 2.5 mM sodium pyrophosphate, 1 mM
503 Na_3VO_4 , 1 mM DTT, 1 mM PMSF 1 μM microcystin, 20 μM benzamidine, and 40 $\mu\text{g/ml}$
504 leupeptin. The detergent-solubilized cell lysates were incubated with anti-FLAG M2 affinity gel

505 (30 μ l per plate, Sigma-Aldrich, A2220) for 1 h at 4 °C, washed four times in lysis buffer and the
506 beads were resuspended in 40 μ l 200 mM Tris, 4 mM DTT, 20 mM $MnCl_2$ for use in *in vitro*
507 phosphatase assay. STAT1 (0.3 μ M) (Biosource, PHF0011) was phosphorylated *in vitro* by
508 incubation with recombinant human cdk1/cyclinB (0.2 μ M) (Millipore, 14-450) at 30 °C for 90
509 min in the presence of 1 mM ATP, and 1 X PK buffer (NEB, B6022) containing 50 mM Tris, 10
510 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 0.01% Brij, pH 7.5, and the reaction was quenched by
511 addition of 144 μ M CDK1 inhibitor RO3306 (Enzo, ALX-270-463). Phosphorylated STAT1
512 substrate was added to 1/4 volume of beads with bound PHLPP1 (or to lysis buffer control) and
513 reactions were allowed to proceed for an additional 120 min at 30 °C. For the zero minute time
514 point, beads were added after the 120 min incubation and all reactions were immediately
515 quenched with 4xSB (sample buffer). Samples were analyzed by western blot.

516 **Mouse infection and endotoxin challenge**

517 Bacterial sepsis in mice was induced by injection of *E. coli* K1 strain RS218 and LPS
518 endotoxemia was induced by injection of purified *E. coli* O111:B4 LPS (Sigma-Aldrich). The *E.*
519 *coli* culture was grown overnight in Luria broth (LB) medium (Hardy Diagnostics) at 37°C with
520 shaking. The bacterial culture was diluted 1:50 in fresh LB, grown to mid-log phase, washed
521 twice with PBS and reconstituted in PBS to yield the appropriate inoculum. For survival
522 experiments, 10 to 14-week-old female C57BL/6 WT and littermate control *Phlpp1*^{-/-} mice were
523 injected i.p. with 5×10^7 colony forming units (cfu) *E. coli* or 15 mg/kg LPS and mouse survival
524 recorded for 10 days following injection. For measurement of serum IL-6, IL-10 and IL-1 β
525 levels, mice were injected with 10 mg/kg LPS, and at 4, 8, 12 and 24 h after injection, 80 μ l of
526 blood was collected by submandibular bleeding using a lancet into a serum separating blood
527 collection tubes (BD) that were spun at $1500 \times g$ for 10 min to separate serum. Serum cytokines

528 were quantified by specific ELISA (R&D) following the manufacturer's protocol. All protocols
529 for mouse experiments were conducted in accordance with the institutional guidelines and were
530 approved by the Institutional Animal Care and Usage Committee (IACUC) at the University of
531 California, San Diego.

532

533 **SUPPLEMENTAL INFORMATION**

534 Supplemental Information includes Extended Experimental Procedures, four figures, and two
535 tables and can be found with this article online.

536 **AUTHOR CONTRIBUTIONS**

537 K.C.-K., J.D.S., S.U., A.T.K., and G. L. performed the experiments. K.C.-K., J.D.S., and A.C.N.
538 wrote the manuscript. K.C.-K., J.D.S., A.T.K, G. L., S.U., V.N., C.K.G. and A.C.N. conceived
539 the project.

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666

667

668 **FIGURE LEGENDS**

669 **Figure 1**

670 PHLPP1 knock-out mice are protected against sepsis-induced death. **(A)** Survival curve of WT
671 and *Phlpp1*^{-/-} mice i.p. infected with 1x10⁷ cfu of *E. coli*. Values are expressed as percent
672 survival of 15 mice for each genotype. ** p < 0.01 by log-rank test. **(B)** Survival curve of WT
673 and *Phlpp1*^{-/-} mice i.p. injected with 15 mg/kg LPS. Values are expressed as percent survival of
674 16 mice for each genotype. * p < 0.04 by log-rank test. **(C-E)** ELISA showing IL-6 **(C)**, IL-1β
675 **(D)** and IL-10 **(E)** levels in serum at the indicated times after i.p. injection of 10 mg/kg LPS.
676 Data represent mean ± SEM. Statistics analyzed by Student's *t*-test *p < 0.05, **p < 0.01.

677 **Figure 2**

678 Loss of PHLPP1 modulates the expression of inflammatory genes in macrophages. **(A)** Heat map
679 for mRNA-Seq expression of the 1,654 mRNA transcripts regulated greater than two-fold with a
680 FDR < 0.05 in BMDMs isolated from WT or *Phlpp1*^{-/-} animals treated with 100 ng/ml KLA for
681 1, 6 or 24 h. Data represent the log₂ difference between the mRNA expression in *Phlpp1*^{-/-}
682 macrophages compared to wild-type macrophages. **(B)** Gene ontology analysis for the 199
683 elevated (red arrow) or 144 decreased (blue arrow) transcripts in *Phlpp1*^{-/-} macrophages
684 compared to wild-type macrophages. **(C)** *De novo* motif analysis using HOMER
685 (Hypergeometric Optimization of Motif EnRichment) for the 199 promoters corresponding to the
686 genes elevated in the *Phlpp1*^{-/-} macrophages. **(D)** Pie graph showing the percentage of promoters
687 of elevated genes that contain STAT or IRF binding motifs. **(E-G)** Normalized mRNA-Seq
688 values for **(E)** *Cd69* **(F)** *Ifit2* and **(G)** *Gbp5* mRNA in BMDMs isolated from WT or *Phlpp1*^{-/-}
689 animals treated with 100 ng/ml KLA for 0, 1, 6, or 24 h. RPKM – Reads Per Kilobase Million.

690 Values are expressed as mean \pm SEM. * $p < 0.05$ (Student's *t*-test) compared to WT cells. See
691 also Tables S1 and S2.

692 **Figure 3**

693 PHLPP1 controls STAT1 genomic recruitment and STAT1-dependent gene expression. (A-C)
694 Quantitative PCR analysis for (A) *Cd69* (B) *Ifit2* and (C) *Gbp5* mRNA isolated from
695 thioglycollate-elicited peritoneal macrophages treated with control siRNA (siCtl) or siRNA
696 specifically targeting *Stat1* (siStat1) and subsequently treated with vehicle or 100 ng/ml KLA for
697 6 or 24 h. Values are expressed as mean \pm SEM from replicate experiments. * $p < 0.05$ (Student's
698 *t*-test) compared to siCtl treated cells. (D-F) Quantitative PCR analysis of ChIPs for STAT1 at
699 the (D) *Cd69* (E) *Ifit2* and (F) *Gbp5* promoter in BMDMs isolated from WT or *Phlpp1*^{-/-} animals
700 and treated with 100 ng/ml KLA for 0, 1, 6 or 24 h. Values are expressed as mean \pm SEM. * $p <$
701 0.05 (Student's *t*-test) compared to WT cells.

702 **Figure 4**

703 PHLPP1 regulates STAT1 phosphorylation on Ser727. (A) Western blot analysis of primary
704 BMDM from WT or *Phlpp1*^{-/-} mice treated with 100 ng/ml KLA for the indicated times and
705 probed with the indicated antibodies. (B) Ratio of pSTAT1 (S727):total STAT1, pSTAT1
706 (Y701):total STAT1 or phosphoERK (T202/Y204):total ERK normalized to the highest value;
707 data represent the mean \pm SEM of five independent experiments as in (A). ** $p < 0.01$ (Student's
708 *t*-test) compared to WT cells. (C) Western blot analysis of an *in vitro* phosphatase assay of
709 purified and phosphorylated STAT1 and immunoprecipitated FLAG-PHLPP1, incubated for 0
710 and 120 min at 30 °C (on the left). On the right, quantification of pSTAT1 (S727) divided by
711 total STAT1 and normalized to 0 time point. Values are expressed as mean \pm SEM of three

712 independent experiments. ** $p < 0.01$ (Student's *t*-test). **(D)** Western blot analysis of HEK-293T
713 cells over-expressing vector control (Vector) or HA-tagged PHLPP1 and treated with 10 ng/ml
714 IFN γ for 0, 1, 6, or 24 h. **(E)** Graphs represent the quantification of three independent
715 experiments as presented in (D). Values are expressed as mean relative units of pSTAT1 (S727)
716 or (Y701) divided by β -Actin and normalized to vector 0 h \pm SEM. * $p < 0.05$ (Student's *t*-test)
717 compared to vector control expressing cells. See also Figure S1.

718

719 **Figure 5**

720 STAT1 Ser727 phosphorylation and transcriptional activity are insensitive to okadaic acid. **(A)**
721 Western blot analysis of primary BMDMs from WT or *Phlpp1*^{-/-} mice treated with 100 ng/ml
722 KLA for 0 or 30 min followed by treatment with 1 μ M OA or DMSO control for an additional
723 15 min. and probed with the indicated antibodies; pAkt antibody recognizes phosphorylated
724 Thr308 **(B)** Graph represents the quantification of three independent experiments as presented in
725 (A). Values are expressed as the mean \pm SEM of the ratio of pSTAT1 (S727) to total STAT1
726 normalized to the highest value; * $p < 0.05$, ** $p < 0.01$ and n.s.- non-significant (Student's *t*-test).

727 **Figure 6**

728 PHLPP1 suppresses STAT1 transcriptional activity by a mechanism that depends on its catalytic
729 activity and an NLS in its N-Terminal Extension **(A)** Luciferase reporter assay in WT (+/+) and
730 *Phlpp1*^{-/-} (-/-) MEFs over-expressing GAS luciferase reporter and treated with 10 ng/ml IFN γ for
731 0, 1, 6, or 24 h in combination with 1 μ M OA or DMSO control treatment for 15 minutes. Values
732 are expressed as mean of relative light units (RLU) \pm SEM of three independent experiments. * p
733 < 0.05 (Student's *t*-test). See also Figure S1. **(B)** Schematic of HA-tagged PHLPP1 constructs
734 used in this study: the PP2C domain of PHLPP1 (PP2C), nuclear targeted PP2C with NLS (NLS-

735 PP2C), NLS-PP2C with active site residues Asp1210 and Asp1413 residues mutated to Ala
736 (NLS-PP2C DDAA), and full-length PHLPP1 (PHLPP1). (C) Luciferase reporter assay in HEK-
737 293T cells over-expressing GAS luciferase reporter in combination with either vector control
738 (vector, black) or the constructs described in (B) and treated with 10 ng/ml IFN γ for 0, 1, 6, or 24
739 h. Values are expressed as mean RLU \pm SEM of four independent experiments. All data points at
740 24 h were significant against each other except for vector to PP2C, vector to NLS-PP2C DDAA,
741 P1 to NLS-PP2C, and PP2C to NLS-PP2C DDAA. * $p < 0.05$, ** $p < 0.01$ (Student's *t*-test). (D)
742 Schematic showing position and sequence of bipartite NLS in the NTE, and NLS mutants used in
743 this study. (E) HeLa cells over-expressing the constructs used in Figure 6D were stained for HA
744 (green), α -Tubulin (red), and DAPI (blue). Scale bar indicates 15 μ m. (F) The Nuclear to
745 Cytoplasmic ratio was calculated for each construct (300 cells per construct) and values are
746 expressed as mean \pm SEM. All data points were significant against each other except for NLS1
747 to NLS2, and NLS2 to NLS1/2. ** $p < 0.01$, n.s. – non-significant (Student's *t*-test). (G)
748 Luciferase reporter assay in HEK-293T cells over-expressing a GAS luciferase reporter in
749 combination with either vector control (vector, black) or the constructs described in (D) however
750 in the context of a full-length PHLPP1 and treated with 10 ng/ml IFN γ for 0, 1, 6, or 24 h.
751 Values are expressed as mean RLU \pm SEM of eight independent experiments. * $p < 0.05$, ** $p <$
752 0.01, n.s. - non-significant (Student's *t*-test).

753 **Figure 7**

754 (A) Luciferase reporter assay in HEK-293T cells over-expressing GAS luciferase reporter in
755 combination with either vector control (Vector, black), PHLPP1 NTE (NTE, green),
756 PHLPP1 Δ NTE (blue), or PHLPP1 (red) and treated with 10 ng/ml IFN γ for 0, 1, 6, or 24 h.
757 Values are expressed as mean of RLU \pm SEM of five independent experiments. All data points at

758 24 h were significant against each other except for vector to PHLPP1 Δ NTE, and PHLPP1 Δ NTE
759 to PHLPP1. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Student's t -test). (B) Western blot analysis of
760 detergent-solublized lysate of HEK-293T cells transfected with vector control (Vector), HA-
761 tagged NTE of PHLPP1, PHLPP1 with the NTE deleted (P1 Δ NTE) or full-length PHLPP1 (HA-
762 P1) and immunoprecipitated (IP) using HA antibody; blots were probed for co-IP of STAT1 tag
763 using GFP antibody. (C) Quantification of GFP-STAT1 IP divided by HA IP and normalized to
764 HA-NTE IP. Values are expressed as mean \pm SEM of three independent experiments. *** $p <$
765 0.001, **** $p < 0.0001$ (Student's t -test).

766

767 **Figure 8**

768 Proposed model for PHLPP1-dependent suppression of STAT1 activity. Binding of IFN γ to IFN
769 receptors results in their dimerization and phosphorylation, promoting the recruitment of JAK,
770 which phosphorylates STAT1 on Tyr701 by JAK. This promotes the dimerization of STAT1
771 and its translocation into the nucleus where it binds the GAS promoter to allow the transcription
772 of inflammatory response genes. Activity of STAT1 is enhanced by phosphorylation on Ser727.
773 However, PHLPP1, which binds STAT1 via its N-terminal extension, tunes the activity of
774 STAT1 by directly dephosphorylating this site to keep activity finely controlled. Loss of
775 PHLPP1 results in poor resolution of inflammatory response.

776 **Table S1. Related to Figure 2.**

777 List of 199 KLA-induced genes that are elevated in *Phlpp1*^{-/-} BMDMs compared to WT cells.

778 **Table S2. Related to Figure 2.**

779 List of 144 KLA-induced genes that are reduced in *Phlpp1*^{-/-} BMDMs compared to WT cells.

780

781 **Figure S1 related to Figure 6.**

782 STAT1 phosphorylation and transcriptional activity are insensitive to okadaic acid. Western blot
783 analysis of extracts used in the luciferase reporter assay in WT (+/+) and *Phlpp1*^{-/-} (-/-) MEFs
784 over-expressing GAS luciferase reporter and treated with 10 ng/ml IFN γ for 0, 1, 6, or 24 h in
785 combination with 1 μ M OA or DMSO control treatment for 15 minutes. Phosphorylation of the
786 PP2A target site on Akt (Thr308) was increased upon OA treatment.

787 **Figure S2 related to Figure 6.**

788 The phosphatase activity of PHLPP1 is important for the regulation of STAT1 activity. (A) HeLa
789 cells over-expressing the HA-tagged constructs used in Figure 6B were stained for HA (green),
790 α -Tubulin (red), and DAPI (blue). Scale bar indicates 15 μ m. (B) The Nuclear to Cytoplasmic
791 ratio was calculated for each construct and values are expressed as mean \pm SEM of: 169 cells for
792 P1, 101 cells for PP2C, 100 cells for NLS-PP2C, and 101 cells for NLS-PP2C DDAA.

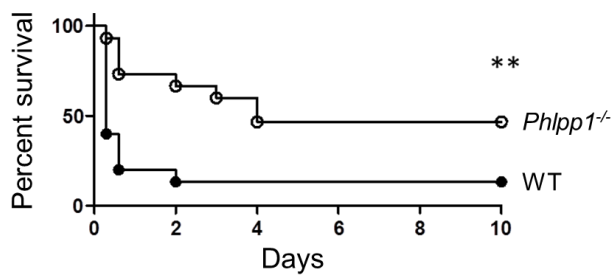
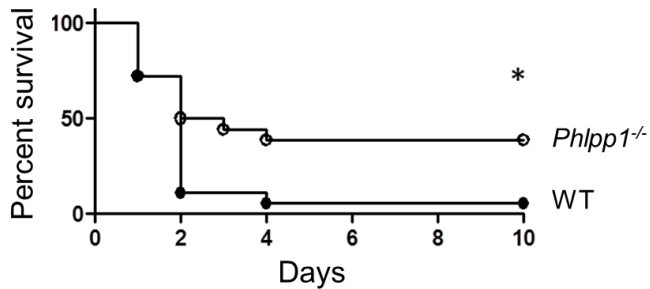
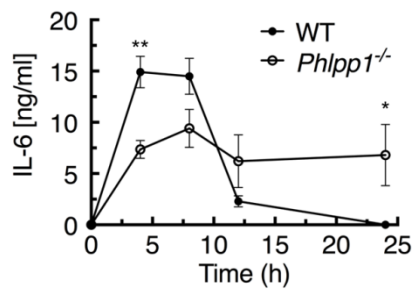
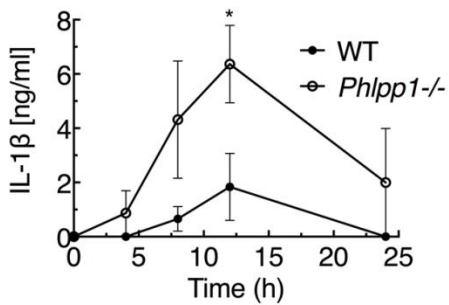
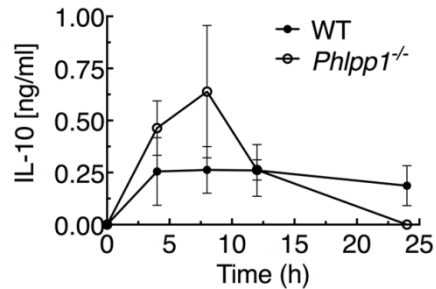
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794 **Figure S3. Related to Figure 6.**

795 PHLPP1 has an NES. (A) Schematic showing position of NES (residues 1121-1134)
796 immediately following the last LRR. (B) HeLa cells over-expressing PHLPP1, the NTE from
797 WT PHLPP1 (NTE-WT) or the NTE-WT in which the PHLPP1 NES was fused to the N-
798 terminus (^{PHLPP1}NES-NTE) were stained for HA (green), α -Tubulin (red), and DAPI (blue). Scale
799 bar indicates 15 μ m.

800 **Figure S4. Related to Discussion.**

801 Luciferase reporter assay in HEK-293T cells over-expressing GAS luciferase reporter and
802 treated with 10 ng/ml IFN γ for 0, 1, 6, or 24 h followed by 250 nM Gö6983 for 10 min, 1 μ M
803 staurosporine for 30 min, or DMSO control. Values are expressed as mean of RLU \pm SEM of
804 three independent experiments.
805

A***Escherichia coli* infection****B****LPS injection****C****D****E****Figure 1**

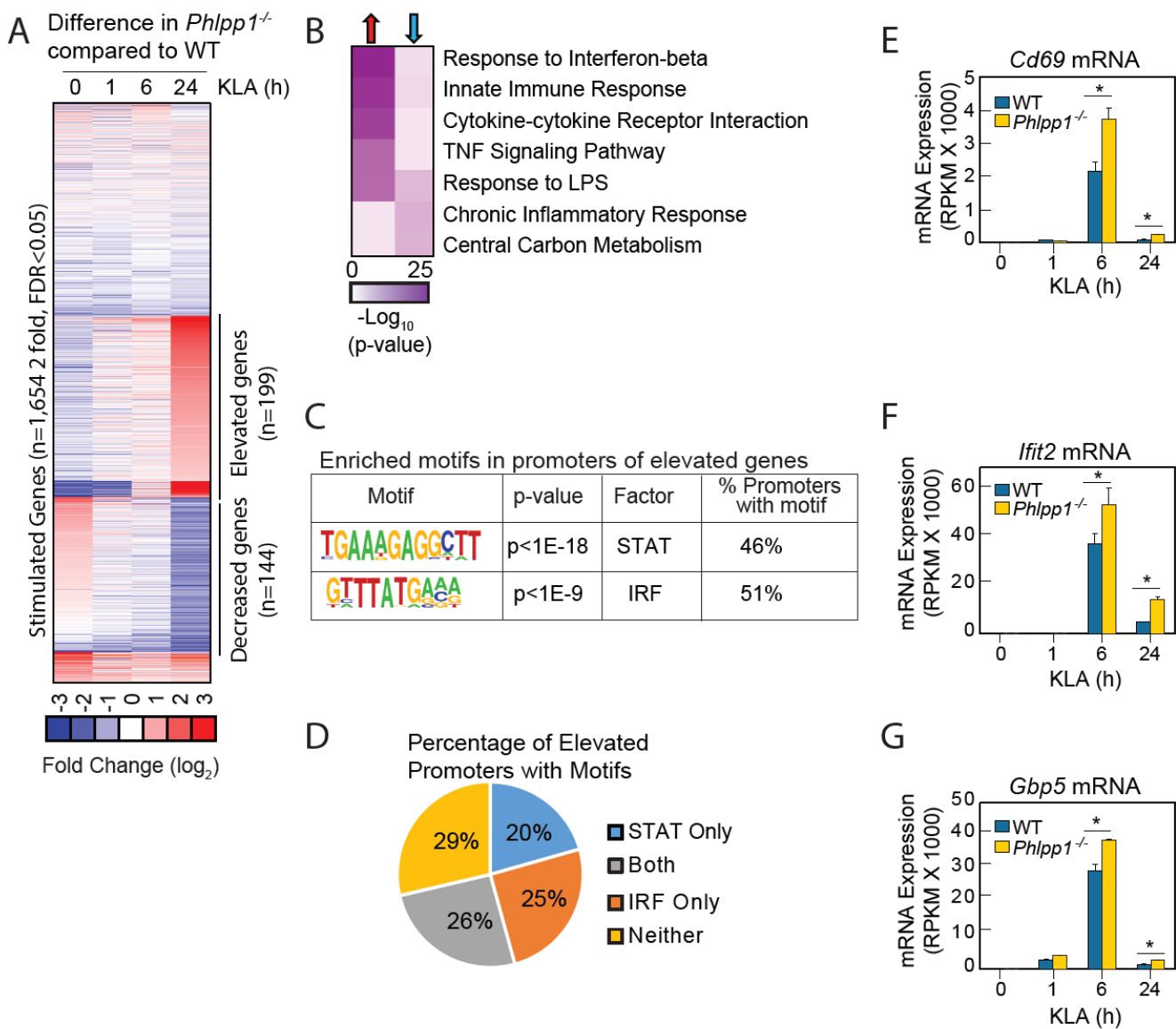


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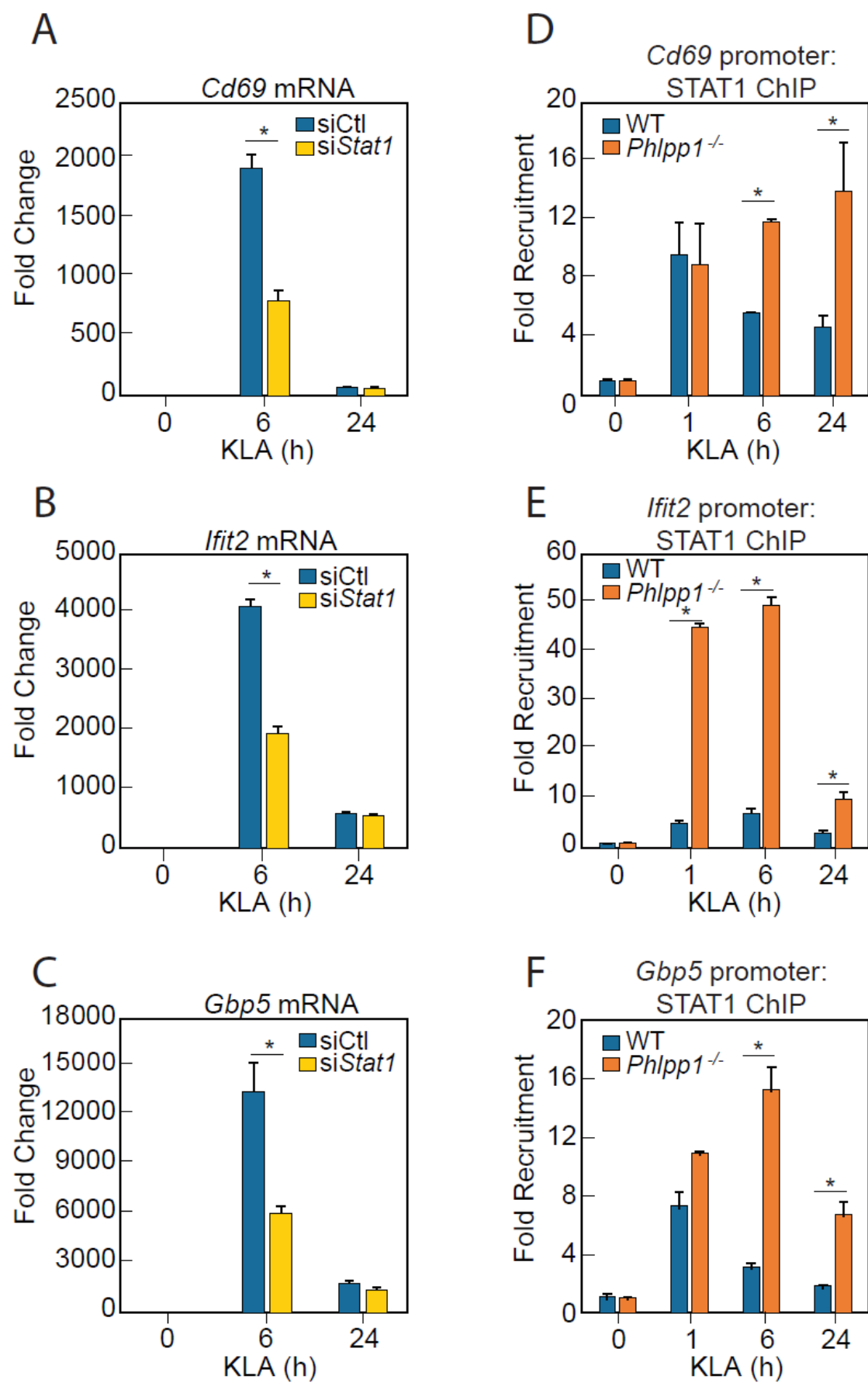


Figure 3

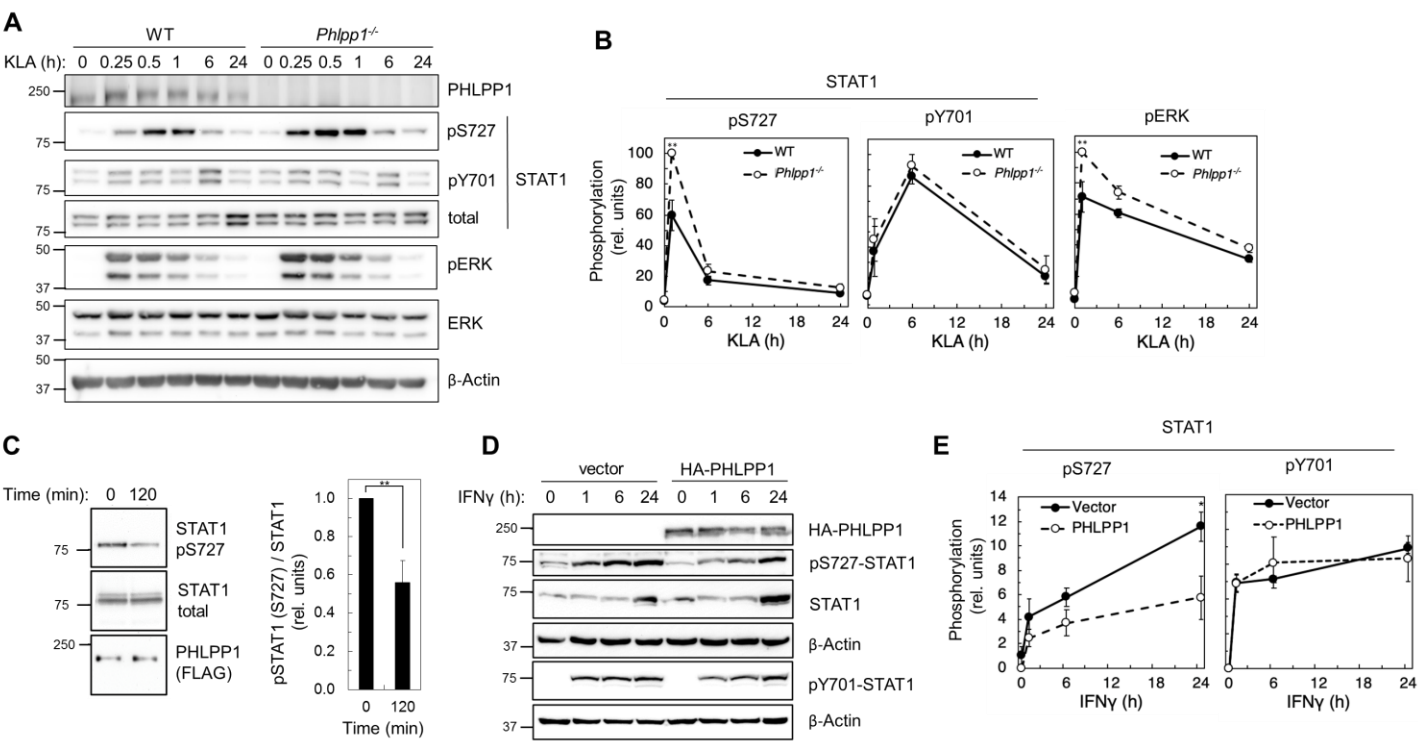


Figure 4

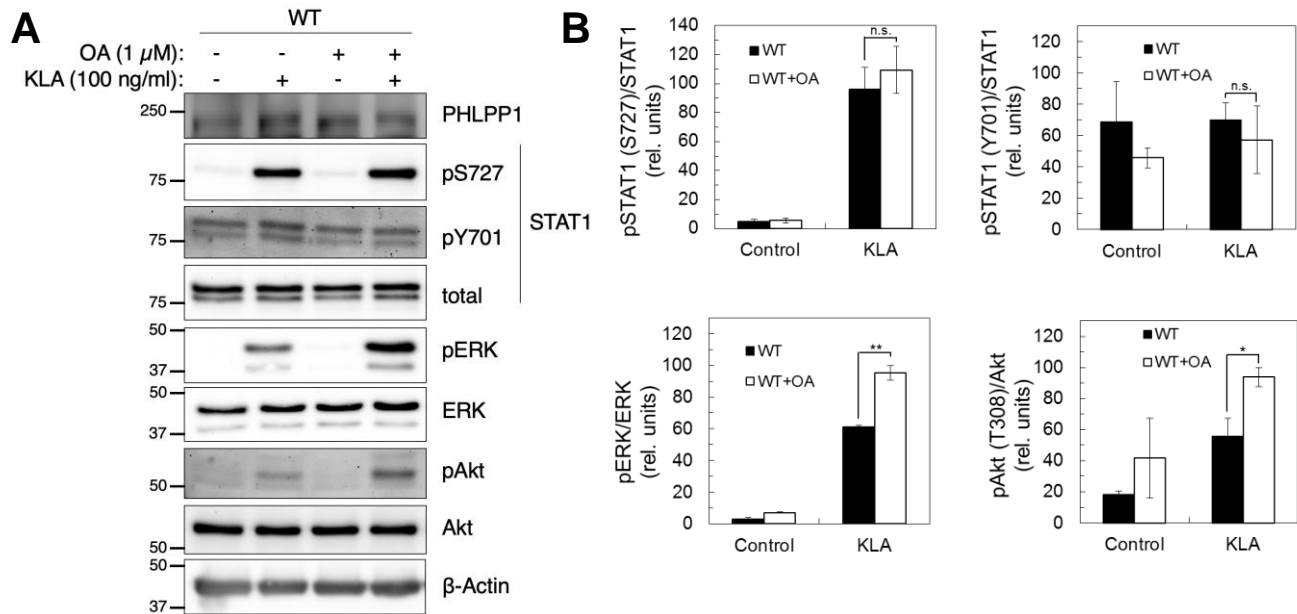


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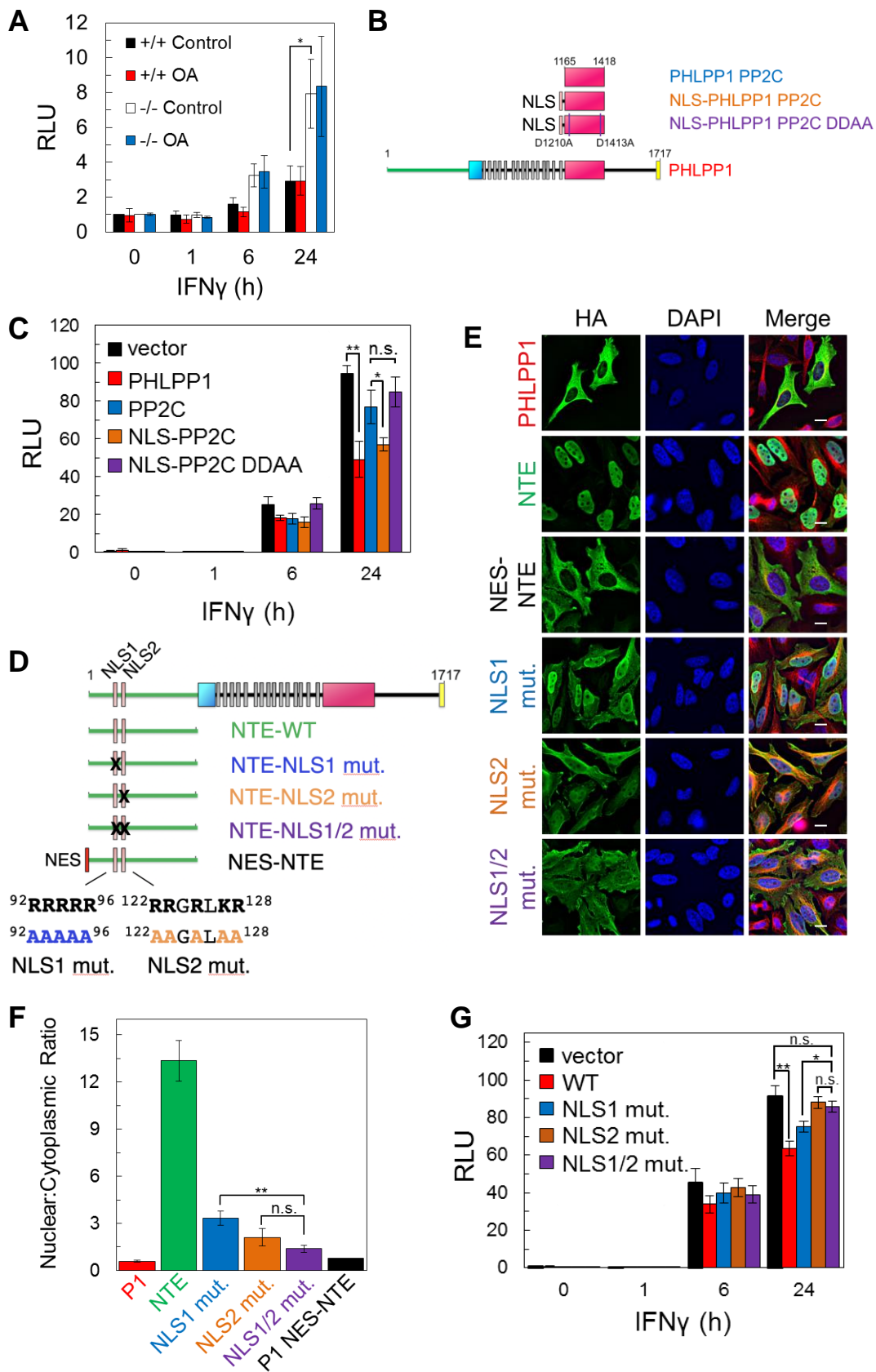


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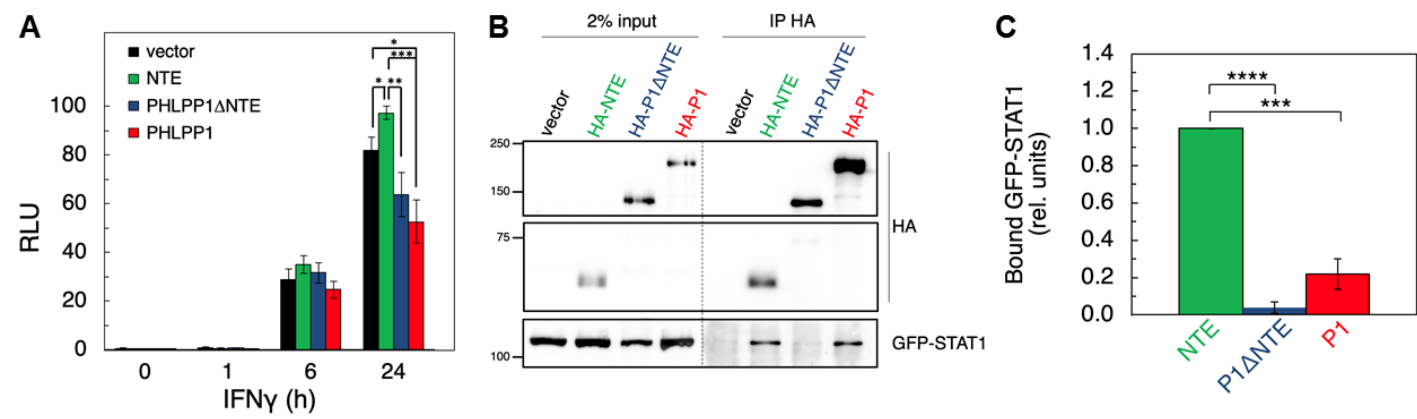


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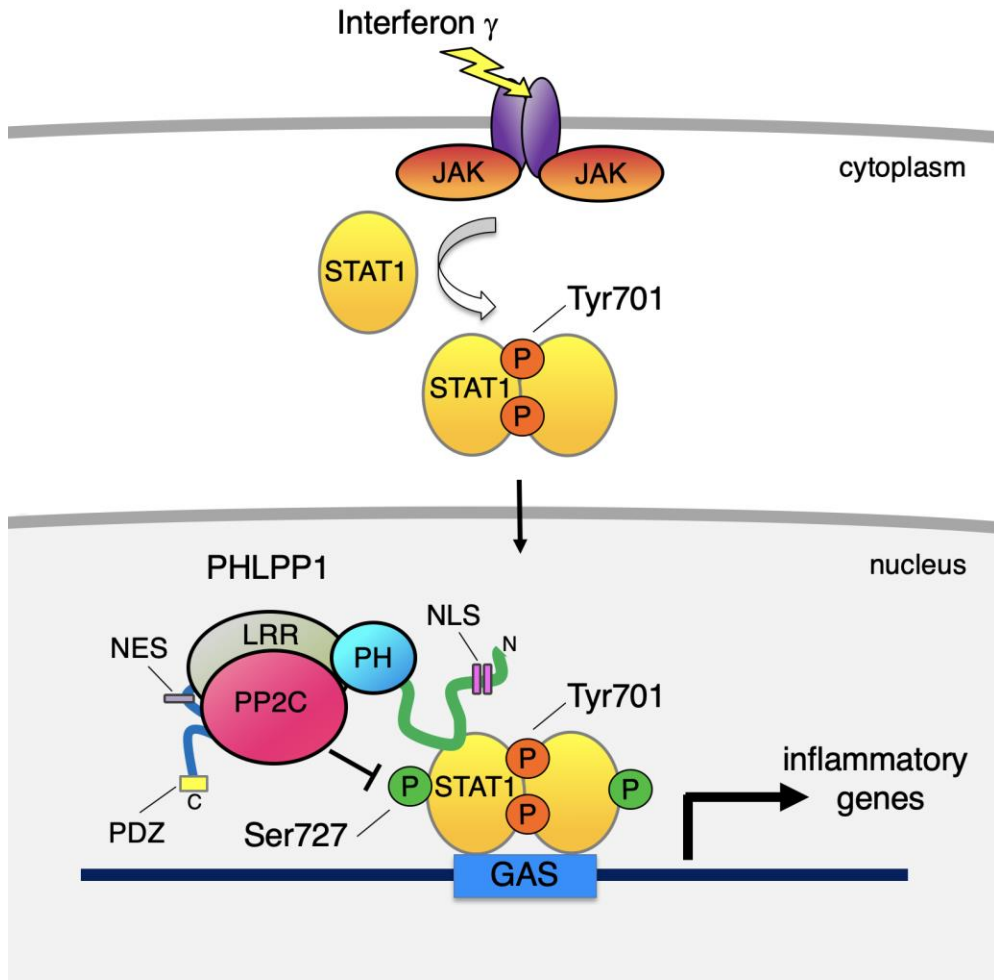


Figure 8

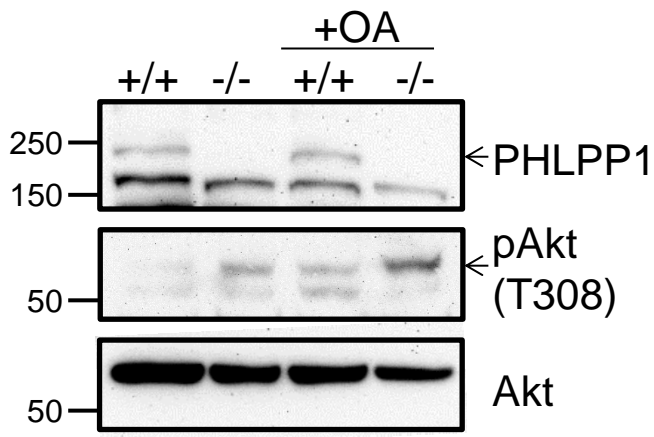
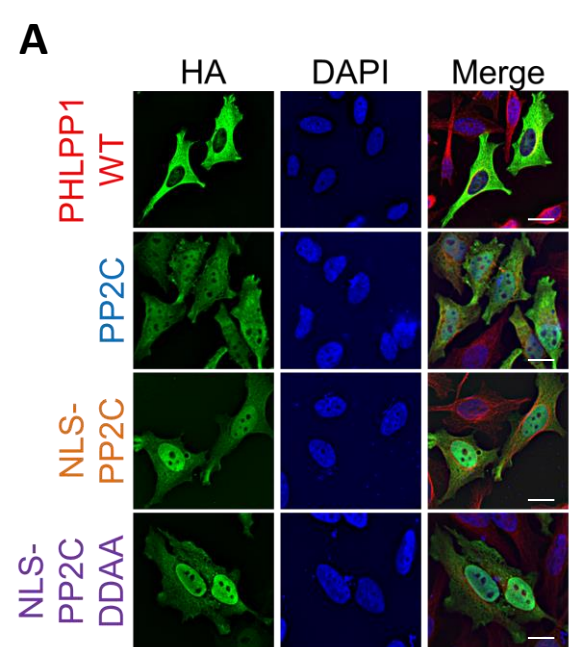


Figure S1



B

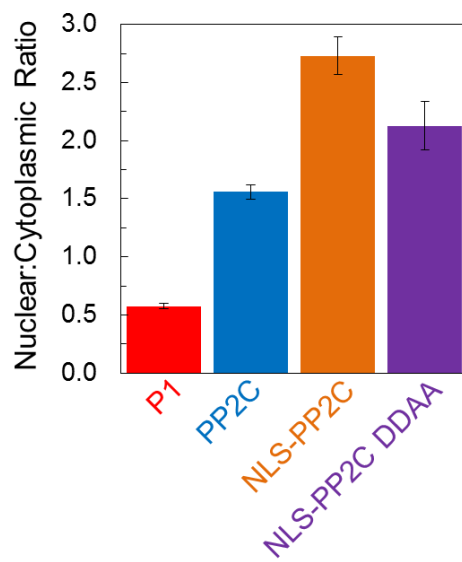
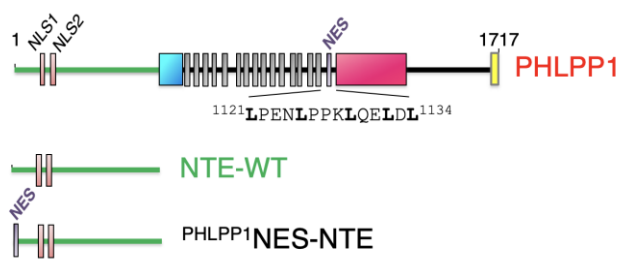
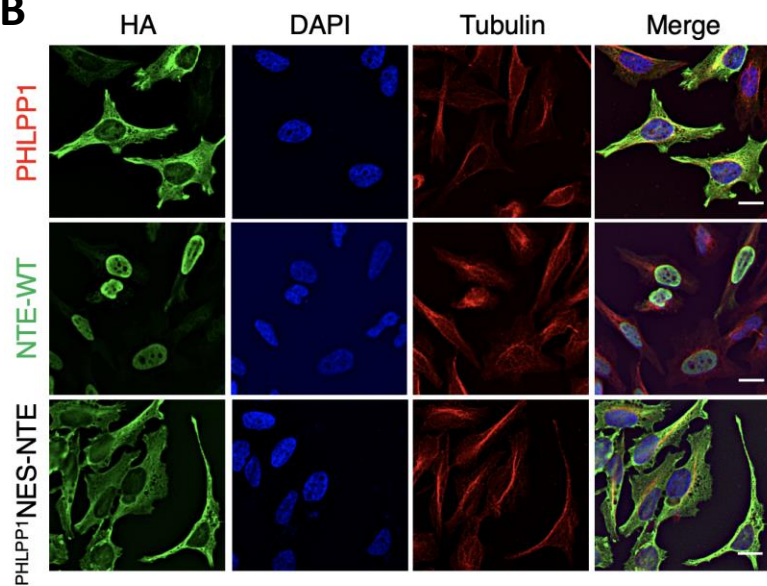


Figure S2

A**B****Figure S3**

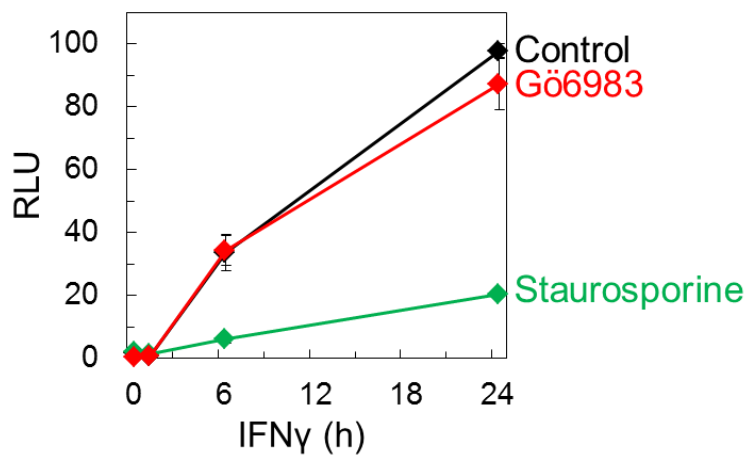


Figure S4