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1 **Serine 165 Phosphorylation of SHARPIN regulates the** 2 **Activation of NF- κ B**

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5 An Thys¹, Kilian Trillet¹, Sara Rosinska¹, Audrey Gayraud¹, Tiphaine Douanne¹, Yannic
6 Danger², Clotilde Renaud¹, Luc Antigny¹, Régis Lavigne^{3,4}, Charles Pineau^{3,4}, Emmanuelle
7 Com^{3,4}, Franck Vérité², Julie Gavard^{1,5}, Nicolas Bidère^{1,6}

8
9 ¹ CRCINA, Inserm, CNRS, Université de Nantes, Université d'Angers, Nantes, France

10 ² Etablissement Français du Sang (EFS), PFBI, Rennes

11 ³ Univ Rennes, Inserm, EHESP, Irset (Institut de recherche en santé, environnement et
12 travail) - UMR_S 1085, Rennes, France

13 ⁴ Protim, Univ Rennes, Rennes, France

14 ⁵ Integrated Center for Oncology, ICO, St. Herblain, France

15 ⁶ Lead Contact

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25 **Abstract**

26 The adaptor SHARPIN composes, together with the E3 ligases HOIP and HOIL1, the linear
27 ubiquitin chain assembly complex. This enzymatic complex catalyzes and stamps atypical
28 linear ubiquitin chains onto substrates to modify their fate, and has been linked to the
29 regulation of the NF- κ B pathway downstream most immunoreceptors, inflammation and cell
30 death. However, how this signaling complex is regulated is not fully understood. Here, we
31 report that a portion of SHARPIN is constitutively phosphorylated on the serine in position
32 165 in lymphoblastoid cells, and can be further induced following antigen receptor
33 stimulation. Analysis of a phosphorylation-resistant mutant of SHARPIN revealed that this
34 mark is dispensable for the generation of linear ubiquitin chains. However, phosphorylation
35 allows the optimal activation of NF- κ B in response to TNF α and T-cell receptor engagement.
36 These results identify a new layer of regulation of the LUBAC, and unveil new strategies to
37 modulate its action.

38

39 Introduction

40 The linear ubiquitin chain assembly complex (LUBAC) is an enzymatic triad of the adaptor
41 SHARPIN (SHANK-associated RH domain-interacting protein) and the E3 ligases HOIP
42 (HOIL-1 interacting protein, also known as RNF31) and HOIL-1 (RanBP-type and C3HC4-
43 type zinc finger-containing protein 1, also called RBCK1, HOIL-1L) [1–3]. This unique
44 complex catalyzes the formation and attachments of atypical linear ubiquitin chains on
45 substrates, thereby modifying their fate. The LUBAC acts as a linchpin by transducing
46 signals from most immunoreceptors to NF- κ B, and therefore emerges as a key regulator of
47 innate and adaptive immunity [1–13]. For instance, the binding of tumor necrosis factor α
48 (TNF α) to its cognate receptor TNFR1 (TNF receptor 1) drives the recruitment of the LUBAC
49 into the so-called complex I. By decorating the key protein kinase RIPK1 (receptor-interacting
50 protein 1 kinase) with linear ubiquitin chains, the LUBAC stabilizes and favors downstream
51 NF- κ B signaling [1–3,7]. Mice deficient in SHARPIN, HOIP or HOIL-1 are hallmarked by an
52 exacerbated TNF α induced cell death [1–3,14–16]. The loss of LUBAC components
53 destabilizes this TNFR signaling complex I and induces the assembly of cytosolic complex II,
54 causing cell death by apoptosis or necroptosis [1,14,15]. While, mice carrying a spontaneous
55 SHARPIN-null mutation (*cpdm*) develop multi-organ inflammation and chronic proliferative
56 dermatitis HOIP and HOIL-1 deficiency is embryonically lethal [1–3,14–16]. The LUBAC also
57 functions downstream of antigen receptors to ensure the optimal activation of NF- κ B, and
58 this signaling pathway is pirated in the activated B cell like subtype of diffuse large B-cell
59 lymphomas (ABC DLBCL) [17–20]. Accordingly, the targeting of the LUBAC was shown to be
60 toxic in ABC DLBCL, unveiling a contribution of this complex to lymphomagenesis [19–22].

61
62 How the LUBAC is regulated continues to be elucidated. All members of the LUBAC have
63 been reported to carry linear ubiquitin chains through auto-ubiquitination [23,24]. Recently,
64 Iwai and colleagues demonstrated that HOIL-1 E3 ligase mono-ubiquitinates the LUBAC,
65 which causes HOIP to preferentially decorate the LUBAC with linear ubiquitin chains rather

66 than other substrates [25]. Linear auto-ubiquitination of the LUBAC can be removed by the
67 OTU deubiquitinase with linear linkage specificity (OTULIN) [23,24]. In addition, HOIP is
68 processed upon TNF α - and TRAIL-induced apoptosis by caspases, with cleaved fragments
69 displaying reduced NF- κ B activation capabilities [26,27]. HOIP is also phosphorylated by
70 mammalian ste20-like kinase 1 (MST1) in response to TNF α , and this modulates its E3
71 ligase activity, thereby attenuating NF- κ B signaling [28]. Three independent groups, including
72 ours, showed that HOIL-1 is cleaved by the paracaspase MALT1 upon antigen receptor
73 engagement and constitutively in ABC DLBCL to allow optimal activation of NF- κ B [29–31].
74 Lastly, SHARPIN is decorated with lysine (K) 63 ubiquitin chains in mice on K312. This
75 ubiquitination was shown to be important for development of regulatory T cells [32].
76 However, what effect K63 ubiquitination of SHARPIN has on NF- κ B signaling still remains an
77 open question. Here, we demonstrate that a fraction of the LUBAC subunit SHARPIN is
78 constitutively phosphorylated in lymphoblastoid cells, and this post-translational modification
79 can be further induced upon antigen receptor engagement. We identify serine (S) 165 to be
80 the primary phosphorylation site of SHARPIN, and provide evidence of its crucial role for the
81 optimal activation of NF- κ B response to both T-cell receptor engagement and TNF α
82 stimulation.

83

84 **Results and discussion**

85 ***SHARPIN is a phosphoprotein***

86 Western blotting analysis of SHARPIN, in human primary CD4⁺ and CD8⁺ cells, Jurkat cells
87 and DLBCL cell lines, revealed that SHARPIN resolves as a doublet (Fig. 1A, EV1A). The
88 treatment of cell lysates with lambda phosphatase, which removes phosphate groups from
89 serine, threonine and tyrosine, effectively chased away the slow migration specie of
90 SHARPIN, suggesting phosphorylation (Fig.1A, EV1A). Conversely, incubating cells with the
91 phosphatase inhibitor Calyculin A resulted in an increase intensity of the slow migration

92 specie of SHARPIN, reinforcing the idea that SHARPIN is indeed phosphorylated (Fig. 1A).
93 Quantification of constitutive SHARPIN phosphorylation in numerous cell lines, revealed that
94 phospho-SHARPIN exists at various levels within different cell types, with lymphoid cells
95 having a relatively high SHARPIN phosphorylation (Fig. 1B). Hence, a fraction of SHARPIN
96 is constitutively phosphorylated.

97

98 Next, we determined whether stimuli, that employ SHARPIN, modify its phosphorylation.
99 Mimicking antigen receptor engagement with phorbol 12-myristate 13-acetate (PMA) plus
100 ionomycin increased the level of SHARPIN phosphorylation, which was efficiently removed
101 by lambda phosphatase (Fig. 1C-D). This was however not the case for all activating stimuli.
102 The change in SHARPIN phosphorylation was induced by PMA, but not ionomycin alone, cell
103 stress inducer Anisomycin or TNF α stimulation (Fig. 1E, EV1B). SHARPIN phosphorylation is
104 therefore specifically increased upon antigen receptor engagement. By comparing the
105 signaling pathways ignited by these stimuli, we noticed that ERK activation was correlated
106 with stimulation-mediated SHARPIN phosphorylation (Fig. 1E, EV1B). Incubating cells with
107 the MEK1/2 inhibitor Trametinib prior to antigen receptor engagement, via PMA plus
108 ionomycin or CD3 plus CD28 stimulation, brought SHARPIN phosphorylation back to basal
109 levels, but did not prevent this constitutive mark (Fig. 1F, EV1C). By contrast, inhibition of the
110 NF- κ B pathway with the PKC inhibitor Bisindolylmaleimide VIII (BIMVIII) had no effect (Fig.
111 1F, EV1C). To gain molecular insights, HOIP, SHARPIN and ERK1-FLAG were
112 overexpressed in HEKT293T cells. Co-immunoprecipitation of FLAG-tagged ERK1 showed
113 an interaction between SHARPIN and ERK1, which was lost in the absence of HOIP (Fig.
114 EV1D). Likewise, SHARPIN phosphorylation could be induced by recombinant ERK1 when
115 the LUBAC is isolated through pulled down of the HOIL-1 subunit (Fig. EV1E). This indicates
116 that SHARPIN requires the LUBAC complex in order to be phosphorylated by ERK1.
117 However, while incubating cell lysates with lambda phosphatase entirely removed SHARPIN
118 phosphorylation, ERK1/2 inhibition only brings SHARPIN phosphorylation back to control
119 conditions (Fig. 1A, 1F). Hence, it seems that at least two kinases are involved in the

120 phosphorylation of SHARPIN, one, yet unidentified, that phosphorylates SHARPIN in resting
121 conditions, and ERK1/2, which phosphorylates SHARPIN upon antigen receptor
122 engagement.

123

124 ***Serine 165 is the major phospho-acceptor site within SHARPIN***

125 Mass spectrometry of SHARPIN, purified by SHARPIN immunoprecipitation of untreated
126 Jurkat cells, was conducted to identify putative phosphorylation sites. In total, 20 peptides
127 were recognized as SHARPIN, covering 45.5% of the protein sequence (Fig. EV2A). Five
128 phosphorylated peptides were identified, and five phosphorylation sites were detected on
129 S129, S131, S146, S165 and S312, with a probability of 65.20%, 50%, 92.44%, 100% and
130 89.49%, respectively (Fig. 2A, EV2B). Out of the 5 serines identified, S165 and S312 were
131 the most conserved ones across species (Fig. 2B). To explore the details of SHARPIN
132 phosphorylation, we engineered a stable Jurkat cell line deficient in *Sharpin* using the
133 CRISPR/Cas9 technology (Fig. EV2C-D). Jurkat *Sharpin* knockout cells were subsequently
134 rescued with an empty vector (EV), wild type (WT) SHARPIN, or phospho-dead mutants of
135 putative SHARPIN phosphorylation sites (S165A, S312A, S165A+S312A (2SA)). S165
136 SHARPIN and 2SA SHARPIN mutants, in both unstimulated and stimulated conditions, had
137 a faster migration in SDS-PAGE of SHARPIN than the WT or S312A SHARPIN (Fig. 2C,
138 EV2E). This implicates S165 to be the main phosphorylation site of SHARPIN. Western
139 blotting analysis with a phospho-specific S165 (p-S165) SHARPIN antibody, after
140 immunoprecipitation of SHARPIN, confirmed that SHARPIN is constitutively phosphorylated
141 and that this phosphorylation can be further induced upon PMA plus ionomycin activation
142 while it remains unaffected by TNF α stimulation (Fig. 2D).

143

144 As previously reported [1–3,14,33], the stability of LUBAC is compromised in SHARPIN
145 deficient cells, which results in a diminished NF- κ B activation as observed by NF- κ B reporter
146 luciferase assay (Fig. EV2D, EV2F). Rescuing Jurkat *Sharpin* knockout cells with WT, S165A

147 or phospho-mimetic S165D SHARPIN resulted in a replenishment of the LUBAC
148 components HOIP and HOIL-1 (Fig. 2E), in a similar manner than the parental cells (Fig.
149 EV2G). Hence, SHARPIN phosphorylation had seemingly no effect on the composition of the
150 LUBAC (Fig. 2F).

151

152 ***SHARPIN phosphorylation contributes to the optimal activation of NF- κ B***

153 The LUBAC is recruited to the TNFR1 complex I upon TNF α stimulation, where it is crucial
154 for stabilizing the complex and subsequent NF- κ B activation. It does so by modifying
155 components of the TNFR1 complex I, including RIPK1, with linear ubiquitin (Methionine-1,
156 M1) chains. This allows for a more efficient recruitment and retention of the I κ B kinase (IKK)
157 complex, consisting of IKK α , IKK β and NEMO, with NEMO itself being modified with M1-
158 chains. When activated, the IKK complex phosphorylates NF- κ B inhibitors I κ Bs leading to
159 their degradation. This liberates NF- κ B to translocate into the nucleus where it can fulfill its
160 transcription factor function [1–3,7,34]. Loss of LUBAC components leads to a switch from
161 complex I to complex II, thereby inducing cell death by apoptosis or necroptosis [1,14,15].
162 We therefore studied the effect of SHARPIN phosphorylation on cell viability following TNF α
163 stimulation. As previously shown [1–3], TNF α stimulation of *Sharpin* knockout cells
164 reconstituted with an empty vector effectively reduced cell viability, while reconstitution with
165 WT-, S165A-, or S165D-SHARPIN resulted in a similar protective effect against TNF α
166 (Fig.3A). Even though phosphorylated SHARPIN is detected at the TNFR1, upon TNF α -
167 FLAG stimulation (Fig. EV3A), recruitment of the phospho-dead SHARPIN to the TNFR1
168 complex I was normal, as was M1 ubiquitination at the TNFR1 receptor (Fig. EV3B).
169 Likewise, TNF α stimulation did not drive any significant changes in total M1 ubiquitination or
170 linear ubiquitination of the known substrate RIPK1 (Fig. 3B). Activation of immune cells by
171 antigen receptor engagement leads to the assembly of a signaling complex of CARMA1,
172 BCL10 and MALT1 (coined CBM complex). This complex serves as docking site for the
173 LUBAC, which authorizes the recruitment and activation of IKK [17–19,33,35]. Within this

174 signaling platform, the paracaspase MALT1 cleaves HOIL-1 [29–31]. We found that the
175 phosphorylation status of SHARPIN did not influence the capability of MALT1 to cleave
176 HOIL-1 upon antigen receptor engagement (Fig. 3C), implicating a normal assembly of the
177 CBM complex. Furthermore, I κ B α phosphorylation was unchanged in cells stimulated with
178 TNF α or with PMA plus ionomycin (Fig. EV3C-D). Altogether; these results suggest that
179 SHARPIN does not affect the recruitment of the LUBAC to the TNFR1 or CBM complexes,
180 nor its ability to catalyze M1 chains and promote IKK activation.

181
182 Nonetheless, SHARPIN phosphorylation emerged as a key player in the optimal activation of
183 NF- κ B (Fig. 3D-G). NF- κ B family members control the transcription of cytokines, and
184 regulate cellular differentiation, survival and proliferation [36]. The NF- κ B family is composed
185 of five related transcription factors in mammals: p50, p52, p65 (also called RelA), c-Rel and
186 RelB [37]. As expected, the transcription activity of NF- κ B, as measured by a NF- κ B reporter
187 luciferase assay, was reduced in *Sharpin* knockout cells, reconstituted with an empty vector,
188 stimulated through antigen receptor engagement or TNF α , when compared to cells
189 expressing WT-SHARPIN (Fig. 3D). Interestingly, NF- κ B activity was restored in cells
190 expressing S165D-SHARPIN, but not S165A-SHARPIN (Fig. 3D), unveiling a key role for
191 S165 phosphorylation. Likewise, cells expressing S165A-SHARPIN displayed a reduced
192 DNA binding of the NF- κ B subunits p65 and p50 upon antigen receptor engagement or TNF α
193 stimulation (Fig. 3E). Of note, the binding of c-Rel was unchanged (Fig. EV3E), whereas p52
194 and RelB signals did not reach the threshold limits (data not shown). In keeping with these
195 results, PCR array of human NF- κ B signaling targets showed a significant diminished
196 expression of numerous cytokines (CSF3, IL12B, IFNBA, IL6, CXCL9, CCL22, IL2 and IL4)
197 and chemokines (CXLCL1, CXCL2, CXCL8, CCL11) in S165A-SHARPIN expressing cells
198 treated with PMA and ionomycin (Fig. 3F, Fig. EV3F). Similar results were found when cells
199 were stimulated with TNF α , albeit only the chemokine receptor CXCR2 reached statistical
200 significance there is a trend towards reduced expression of other NF- κ B signaling targets

201 (Fig. 3G, EV3G). It should be mentioned that compared to PMA plus ionomycin stimulation,
202 induction of NF- κ B target genes by TNF α was weaker, which may also explain why
203 differences upon TNF α stimulation are less striking. Nevertheless, we established that the
204 phosphorylation of SHARPIN on S165 is participates in NF- κ B upon antigen receptor
205 engagement and TNF α stimulation.

206

207 In summary, we have discovered that a part of SHARPIN is constitutively phosphorylated in
208 lymphoblastoid cells and identified S165 as the main phospho-acceptor residue. We also
209 provide evidence that SHARPIN is further phosphorylated on the Serine 165 by ERK upon
210 antigen receptor engagement, but not in response to TNF α (Fig. 1C). Yet, the constitutive
211 phosphorylation of SHARPIN is pivotal for the optimal activation of NF- κ B in response to
212 antigen receptor engagement and TNFR ligation. This apparent dichotomy militates against a
213 role for the inducible phosphorylation in NF- κ B activation. It is therefore tempting to
214 speculate that ERK-mediated phosphorylation of SHARPIN plays an independent function.
215 Beside its crucial role in NF- κ B signaling, a growing body of literature suggests that
216 SHARPIN also acts as an inhibitor of the integrin adhesion receptors [38–40]. Pouwels *et al.*
217 demonstrated that SHARPIN locates at and controls the detachment of cellular protrusions
218 called uropods in lymphocytes, and that this is essential for lymphocyte movement [39]. As
219 antigen receptor engagement delivers a stop signal to migrating T lymphocytes [41–43],
220 ERK-dependent SHARPIN phosphorylation may have an impact on cell adhesion and
221 migration. This description of at least two kinases targeting the same site, with different
222 outcomes is reminiscent of what has been shown for MLKL (mixed lineage kinase domain-
223 like pseudokinase). MLKL acute phosphorylation by RIPK3 at T357 and S358 triggers
224 necrotic cell death [44], while basal phosphorylation on the same sites by a yet-to-be-defined
225 kinase promotes the generation of small extracellular vesicles [45]. Lastly, the LUBAC may
226 exist in different complexes, with different binding partners [46,47]. One could therefore

227 speculate that different kinases could target different LUBAC complexes, and consequently
228 exert selective functions.

229

230 Although necessary for complete NF- κ B activity upon antigen receptor engagement and
231 TNF α stimulation, how SHARPIN phosphorylation regulates the LUBAC functions and NF- κ B
232 signaling is unclear. The basal S165 SHARPIN phosphorylation appeared dispensable for
233 TNF α -induced cell death, linear ubiquitination, assembly of CBM or TNFR1 complexes, and
234 IKK activation. Additional work is therefore required to better understand this new layer of
235 complexity in the regulation of NF- κ B transcription activity. Nevertheless, this may also open
236 up new avenues for therapeutic prospects of aggressive lymphoma, such as ABC DLBCL,
237 for which the LUBAC and NF- κ B activation is pivotal for survival [19,33]. *Hoip* and *Sharpin*
238 deficiency in mice results in embryonic lethality or multiorgan inflammation, respectively,
239 which is driven by aberrant TNF α -induced cell death [14,15,48–50]. Directly targeting
240 SHARPIN phosphorylation for treatment may therefore circumvent the pitfall of inducing
241 autoinflammatory diseases while targeting the NF- κ B pathway specifically. The identification
242 of the kinase responsible for constitutive SHARPIN phosphorylation will therefore be
243 paramount to our future research.

244

245 **Material and Methods**

246 ***Cell culture***

247 Jurkat E6.1 T lymphocytes, HeLa, HEK293T and MCF7 cells were purchased from American
248 Type Culture Collection (ATCC). U2932, SUDHL4, BJAB, RIVA, OCI-LY3, and OCI-LY19
249 cells were acquired from DSMZ. HBL1, HLY1 and TMD8 cells were kindly provided by Martin
250 Dyer, Pierre Brousset, and Daniel Krappmann, respectively. Blood was obtained from
251 healthy donors (Etablissement Français du Sang). Peripheral blood mononuclear cells
252 (PBMC) were acquired by Ficoll density gradient. Primary CD4⁺ and CD8⁺ T cells were

253 subsequently isolated from PBMC using REAlease CD4 and CD8 microbeads kits, from
254 Miltenyi as manufacturer's instructions. Cell viability was assessed using CellTiter-Glo
255 following manufacturer's instructions (Promega) following stimulation with 10 ng/mL TNF α
256 (R&D systems) for 24h.

257

258 ***Immunoblotting***

259 Jurkat E6.1, PBMC or primary T cells were stimulated with 20 ng/mL Phorbol 12-Myristate 13
260 Acetate (PMA, Merck), 300 ng/mL ionomycin (Merck), PMA plus ionomycin, 1 μ g/mL CD3
261 plus 1 μ g/mL CD28 (Becton Dickinson Biosciences), 12.5 mg/mL anisomycin (Merck), 10
262 ng/mL TNF α (R&D systems) or TNF α -Flag (Enzo Life Sciences). To ensure inhibition of
263 certain signaling pathways Jurkat E6.1, PBMC or primary T cells were pre-treated during 1h
264 with 1 μ M Trametinib (Selleckchem), 500 nM Bisindolylmaleimide VIII (BIMVIII, Enzo), 50 μ M
265 SP6000125 (Cell Signaling), or 5 μ M SB203580 (Selleckchem). Cells were washed with ice-
266 cold PBS and subsequently lysed using two possible lysis buffers, as indicated throughout
267 the manuscript. (1) Cells pellets were incubated with 188 μ l of Buffer A (10 mM HEPES
268 pH7.9, 10 mM KCL, 0.1 mM EDTA, 0.1 mM EGTA, 1mM DTT and 1 mM Na₃VO₄) for 5 min
269 on ice. 12 μ l of Buffer A containing 10% Igepal was added for another 5 min on ice and
270 samples were subsequently centrifuged at 1,000 g for 3 min. Supernatant was collected. (2)
271 Cell pellets were lysed with TNT buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton
272 X-100, 1% Igepal, 2 mM EDTA), and incubated for 30 min on ice. Extracts were cleared by
273 centrifugation at >10 000 x g. Lysis buffers were supplemented with 1x Halt Protease
274 Inhibitor cocktail (ThermoFisher Scientific). Protein concentration was determined using the
275 Micro BCA Protein Assay kit (ThermoFisher scientific), for both lysis buffers. Lambda
276 phosphatase experiments used either Buffer A or TNT lysis without EDTA or EGTA for cell
277 lysis. 10 μ g of protein was incubated with 600 Units of lambda phosphatase, 1x NEB buffer
278 for Protein Metallophosphatase and 1mM MnCl₂ (New England Biolabs) for 30 min at 30°C.

279 10 µg of proteins was incubated with 2X Laemmli buffer (Life Technologies) at 95°C for 3
280 min, separated by SDS-PAGE using a 3-15% Tris-Acetate gel and transferred to
281 nitrocellulose membranes (GE Healthcare). Densitometry analysis was performed by the
282 Image J software (National Institutes of Health).

283 The following antibodies were used for analysis by immunoblot: SHARPIN (A303-559A),
284 HOIP (A303-560A), and IκBβ (A301-828A) antibodies were purchased from Bethyl. HOIL-1
285 (sc-393754), αTubulin (sc-8035), CYLD (sc-137139) and GAPDH (sc-32233) antibodies were
286 obtained from Santa Cruz Biotechnology. Phospho-ERK (#9106), IκBα (#9242), phospho-
287 IκBα (#9246), phospho-p38 (#9215), phospho-JNK (#9255) and RIP1 (#3493) were acquired
288 from Cell Signaling Technologies. Anti-linear Ubiquitin (MABS199) was procured from
289 Millipore. For detection of the phospho-S165 from of human SHARPIN, five Balb/C mice
290 were primed via intraperitoneal injection with complete Freund adjuvant and boosted two
291 times at two-week intervals with incomplete Freund adjuvant with a synthetic phosphorylated
292 peptide (DLPR(Sp)PGNLTERC) conjugated to KLH. Mouse blood was collected from the
293 submandibular vein and serum reactivity was confirmed by ELISA with phosphorylated and
294 non-phosphorylated biotinylated peptide. Mouse serum was used to reveal phospho-S165
295 SHARPIN.

296

297 ***Immunoprecipitation (IP) and ERK recombinant kinase assay***

298 Cells were washed with ice-cold PBS, lysed with TNT buffer and incubated for 30 min on ice.
299 Extracts were cleared by centrifugation at >10,000 g. Protein concentration was determined
300 using the Micro BCA protein kit. Samples were precleared with Protein G Sepharose (Merck)
301 for 30 min, and subsequently incubated with 1 µg of antibody and protein G Sepharose for a
302 duration of 2h. Flag pull down was performed incubating cleared cell lysates with Anti-flag
303 M2 affinity gel (Merck) during 2h. SHARPIN (A303-559A, Bethyl), HOIL-1 ((sc-393754, Santa
304 Cruz Biotechnology) and linear ubiquitin, kindly provided by VM Dixit (Genentech), antibodies
305 were used for IP. ERK1/2 kinase assay was performed from beads immunoprecipitated for

306 HOIL-1. Beads were washed in kinase buffer (5 mM MOPS pH 7.2, 5 mM MgCl₂, 1 mM
307 EGTA, 0.4 mM EDTA, 0.05 mM DTT). Beads were subsequently incubated with kinase
308 buffer, 2mM ATP, 350 ng/mL active untagged ERK1 (Merck), 350 ng/mL active ERK2-GST
309 tagged (Merck) or 175 ng/mL ERK1 plus 175 ng/mL ERK2-GST for 30 min at 30°C.

310

311 ***Mass Spectrometry***

312 Jurkat E6.1 cells were lysed in TNT lysis buffer supplemented with 1x Halt protease inhibitor
313 cocktail. Immunoprecipitation of SHARPIN was performed as described above. Proteins
314 were separated by SDS-PAGE. The Colloidal Blue Staining kit (LC6025, Invitrogen) was
315 used to stain protein as per manufacturer's instructions. Gel was excised between 37 and 50
316 kDa, to ensure the presence of SHARPIN, and subsequently cut into smaller fragments.
317 Fragments were washed alternating between 100 mM Ammonium Bicarbonate and
318 Acetonitrile. Disulfide bonds of proteins were reduced, with using 65 mM DTT for 15 min at
319 37°C, and subsequently alkylated by 135 mM Iodoacetamide for 15 min at room
320 temperature. Enzymatic digestion of proteins was performed at 37°C overnight by incubating
321 the gel pieces in 25 ng/mL pH 8.5 trypsin solution (Sequenced Grade Modified Trypsin, ref
322 V511A, Porcine, Promega). Digested peptides were extracted by incubation in 70% and
323 100% acetonitrile for 20 min each. Peptide extracts were evaporated and afterward
324 reconstituted in 0.1% formic acid. Peptide extracts were analyzed by liquid nano-
325 chromatography (nanoLC) nanoElute coupled with a TimsTOF Pro mass spectrometer
326 (Bruker), as previous described by [51]. Generated peaks were analyzed with the Mascot
327 database search engine (MatrixScience version 2.5.01) for peptide and protein identification.
328 The peaks were queried simultaneously in the: UniprotKB Human (release 20191016, 20656
329 sequences) database and a decoy database that was interrogated in parallel to estimate the
330 number of false identifications and to calculate the threshold at which the scores of the
331 identified peptides are valid. Mass tolerance for MS and MS/MS was set at 15 ppm and 0.05
332 Da, respectively. The enzyme selectivity was set to full trypsin with one miscleavage allowed.

333 Protein modifications were fixed carbamidomethylation of cysteines, variable oxidation of
334 methionine, variable phosphorylation of serine, threonine or tyrosine. Identified proteins are
335 validated with an FDR < 1% at PSM level and a peptide minimum score of 30, using Proline
336 v2.0 software [52]. Proteins identified with the same set of peptides are automatically
337 grouped together. Analysis of post-translational modifications was also executed using
338 Proline v2.0 Software. Localization confidence values for peptide ions were exported from
339 Mascot [53] and a site probability is calculated for a particular modification according to the
340 number of peptides confidently detected with this site modification.

341

342 ***CRISPR/Cas9 knock out and SHARPIN reconstituted cell lines***

343 LentiGuide and LentiCRISPRv2 vectors (GeCKO, ZhangLab), were cloned to contain
344 SHARPIN guide RNA (5' CACCGTGGCTGTGCACGCCGCGGTG 3'), as described before
345 (Sanjana et al., 2014; Shalem et al., 2014). LentiCRISPRv2, together with the packaging
346 vectors PAX2 and VSV-G, were transfected in HEK293T cells using a standard calcium
347 phosphate protocol, as previously published [54]. Supernatant, containing the lentiviral
348 particles, was collected after 48 h, and used to infect 10^6 Jurkat cells in presence of 8 $\mu\text{g}/\text{mL}$
349 Polybrene (Santa Cruz Biotechnology). Jurkat cells expressing the LentiCRISPRv2 vector
350 were selected by adding 1 $\mu\text{g}/\text{mL}$ Puromycin to their media, and subsequently dilution cloned.
351 Single-cell clones were then picked and tested for functional Cas9 cutting of SHARPIN
352 (Fig.EV2C). SHARPIN was cloned from a pCMV3flag9SHARPIN vector (Addgene), into a
353 pCMH-MSCV-EF1a-puroCopGFP vector (SBI). Site directed mutagenesis was performed
354 using the pCMH-MSCV-EF1a-puroCopGFP vector containing wild type (WT) SHARPIN, to
355 substitute the serine (S) residues on 165 or/and 312 to an alanine (A) or an aspartic acid (D).
356 CRISPR/Cas9 resistance was achieved by site directed mutagenesis of the PAM sequence
357 on site 29, substituting an Arginine (AGG) to an Arginine (AGA).

358

359 ***NF- κ B assays***

360 NF- κ B luciferase assay was performed as previously described [20,29]. DNA binding of the
361 NF- κ B subunits was measured using TransAM NF- κ B activation assay (Actif Motif), as per
362 manufacturer's instructions, with reconstituted Jurkat cells were treated with 20 ng/mL PMA
363 plus 300 ng/mL Ionomycin or 10 ng/mL TNF α during 30 min. For the RT² profiler PCR array
364 of human NF- κ B signaling targets, cells were treated with 20 ng/mL PMA plus 300 ng/mL
365 Ionomycin or 10 ng/mL TNF α for 4h. RNA was extracted using the Nucleospin RNAplus kit
366 (Macherey-Nagel), following manufacturer's instructions. 2 mg of RNA was reverse
367 transcribed using the Maxima Reverse Transcriptase kit (ThermoFisher). RT² profiler PCR
368 array of human NF- κ B signaling targets was performed as instructed by the manufacturer
369 (Qiagen).

370

371 ***Statistical analysis***

372 Statistical analysis, comparing multiple groups was performed using two-way ANOVA on
373 rank test with Tuckey's post hoc test in GraphPad Prism 7 software. TransAM NF- κ B
374 activation assays were analyzed using a 2-way ANOVA test with a Sidak correction for
375 multiple analysis. RT² profiler PCR array was performed using the online software provided
376 by the manufacturer (<https://dataanalysis2.qiagen.com/pcr>). In short, the p-values were
377 calculated based on a student t-test of the replicate $2^{-(DC_T)}$ values for each gene in the
378 control group and treatment group. The p-value calculation used is based on a parametric,
379 unpaired, two sample equal variance, two tailed distribution. P-values < 0.05 were
380 considered as significant.

381

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394 **Author contribution**

395 An Thys: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original
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398 Yannic Danger and Franck Vérité: Resources. Emmanuelle Com: Methodology, Formal
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402

403 **Conflict of interest**

404 The authors declare that they have no conflict of interest.

405

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569

570 **Figure legends**

571 **Figure 1. SHARPIN is a Phosphoprotein.**

572 A Jurkat cells were treated with or without 50 nM Calyculin A (Cal A) for 30 min prior
573 lysis. Cell lysates from primary human CD4⁺ or CD8⁺ T lymphocytes and Jurkat cells
574 were treated with lambda phosphatase (λ PP) as indicated, and subjected to Western
575 blotting analysis. Green and white arrowheads indicate phosphorylated and
576 unphosphorylated SHARPIN, respectively. Molecular weight markers (M_r) are
577 indicated.

578 B Densitometric analysis of the ratio between phosphorylated species of SHARPIN and
579 SHARPIN in primary human T CD4⁺ cells, primary human T CD8⁺ T cells, Jurkat T
580 cells, a panel of patient-derived diffuse large B-cell lymphoma cell lines, HeLa and
581 MCF-7 cells.

582 C-D Primary human CD4⁺ T (E) and CD8⁺ T lymphocytes (F), and Jurkat cells (G) were
583 stimulated for 30 min or as indicated with 20 ng/mL PMA plus 300 ng/mL ionomycin
584 (PI). Lysates were prepared and treated or not with λ PP and Western blotting
585 analysis was performed as indicated.

586 E Jurkat cells were stimulated with 20 ng/mL PMA, 300 ng/mL ionomycin, PMA plus
587 ionomycin (PI), 10 ng/mL TNF α or 12.5 μ g/mL Anisomycin, for 30 min. Cell lysates
588 were subjected to Western blotting analysis.

589 F Jurkat cells we pretreated for 1h with 1 μ M Trametinib or 500 nM BIMVIII and
590 subsequently stimulated and lysed as in (E). Blue arrowhead indicates HOIL-1 Cter.

591

592 **Figure 2. The Serine Residue of SHARPIN in Position 165 is a major Phosphorylation** 593 **Site.**

594 A Mass spectrometry analysis of immunoprecipitates prepared from Jurkat cell lysates
595 with antibodies to SHARPIN identified phosphorylation sites. The identified peptides

596 were validated with an FDR < 1% and a minimum score of 30. The peptide score
597 calculated in the Mascot search engine following $-10\log_{10}(p)$, where p is the absolute
598 probability. The position of the post-translational modification in the protein (PTM), the
599 modification site probability calculated in Proline (site probability) the number of
600 peptides identified, the peptide sequence with the identified amino acid in blue the
601 localization and other amino acid modifications in green.

602 B Sequence alignment of the amino acids surrounding the serine (S) 129, S131, S146,
603 S165 and S312 residues.

604 C Jurkat cells knockout for *Sharpin* were engineered by CRISPR/Cas9. Cells were
605 complemented with an empty vector (EV), or with wild-type (WT), S165A-, S312A- or
606 S165A plus S312A (2SA)-SHARPIN. Cells were stimulated for 30 min with antibodies
607 to CD3 and CD28 (1 μ g/mL each). Cell lysates were prepared and subjected to
608 Western blotting analysis. White and green symbols show SHARPIN and
609 phosphorylated SHARPIN, respectively. Molecular weight markers (M_r) are indicated.

610 D *Sharpin* knockout Jurkat cells reconstituted with EV, WT- or S165A-SHARPIN were
611 treated with PI as in (C), or with 10 ng/mL TNF α . Cells lysates were
612 Immunoprecipitated (IP) with antibodies specific to SHARPIN and subjected to
613 Western blotting analysis with a mouse antibody specific for p-S165-SHARPIN.

614 E Cell lysates from *Sharpin* knockout Jurkat cells reconstituted with an empty vector
615 (EV), wild-type (WT-) or S165A-SHARPIN were subjected to Western blotting
616 analysis with antibodies specific to the indicated antibodies.

617 F Cell lysates from EV, WT-, S165A- or S165D-SHARPIN reconstituted *Sharpin*
618 knockout Jurkat cells were submitted to SHARPIN immunoprecipitation (IP) prior to
619 western blotting analysis for LUBAC components. * indicates the heavy chain of the
620 SHARPIN antibody used for IP.

621

622 **Figure 3: Serine 165 Phosphorylation of SHARPIN is crucial for the optimal Activation**
623 **of NF- κ B.**

- 624 A *Sharpin* knockout Jurkat cells expressing an empty vector (EV) or wild type (WT-),
625 S165A- or S165D-SHARPIN were stimulated with 10 ng/mL TNF α for 24 h. Cell
626 viability was assessed with CellTiter glo (mean \pm SEM; ***P<0.001 by two-way
627 ANOVA).
- 628 B WT- and S165A-expressing Jurkat cells were stimulated with 10 ng/mL TNF α as
629 indicated. Cell lysates were subjected to immunoprecipitation (IP) of linear ubiquitin
630 (M1) prior to Western blotting analysis with the specified antibodies. Molecular weight
631 markers (M_r) are indicated.
- 632 C *Sharpin* knockout Jurkat cells expressing an empty vector (EV) or wild type (WT-) or
633 S165A-SHARPIN were stimulated with 20 ng/mL PMA plus 300 ng/mL ionomycin (PI)
634 or CD3 plus CD28 (1 μ g/mL each) for 30 min. Cell lysates were subjected to western
635 blotting analysis. Blue symbol indicates HOIL-1 Cter cleavage band.
- 636 D NF- κ B reporter luciferase assay in the indicated Jurkat cells treated for 6 h with 20
637 ng/mL PMA plus 300 ng/mL ionomycin (PI), 1 μ g/mL anti-CD3 plus 1 μ g/mL anti-
638 CD28, or with 1 ng/mL TNF α . Graph displays 1 out of 3 experiments done in triplicate
639 (mean \pm SEM; *p<0.05, **P<0.01 by two-way ANOVA).
- 640 E WT- or S165A-SHARPIN reconstituted Jurkat cells were stimulated with PI or TNF α
641 for 30 min as in (D) before performing TransAM NF- κ B activation assay for p65 and
642 p50 NF- κ B subunits. The presented Graph show 1 out of 3 experiment performed in
643 duplicate (mean \pm SEM; *p<0.05, **P<0.01 by two-way ANOVA).
- 644 F-G Volcano plot or RT² profiler PCR array of human NF- κ B signaling targets for cells
645 stimulated for 4 h with PI (F) or TNF α (H). (G) Genes indicated on the volcano plot
646 are significantly down regulated with a fold change > 2 in cells expressing S165A-
647 SHARPIN when compared to WT-SHARPIN. On (G), CXCR2 is significantly down
648 regulated with a fold change >2, while other genes indicated are borderline significant
649 in S165A-SHARPIN expressing cells, compared to WT-SHARPIN. (F-G) Heatmap in

650 the below volcano plot gives an overall view of the gene expression in the RT² profiler
651 PCR array.

652

653 **Expanded View Figure legends**

654 **Figure EV1, relative to Figure 1 SHARPIN Phosphorylation by ERK upon Antigen**
655 **Receptor Engagement.**

656 A Western blotting analysis of a panel of patient-derived diffuse large B-cell lymphoma
657 (DLBCL) cell lines. Cell lysates were treated with lambda phosphatase (λ PP) as
658 indicated, and subjected to Western blotting analysis. Green and white arrowheads
659 indicate phosphorylated and unphosphorylated SHARPIN, respectively. Molecular
660 weight markers (M_r) are indicated.

661 B Primary peripheral blood mononuclear cells (PBMC) were stimulated with 12.5 μ g/mL
662 Anisomycin, 20 ng/mL PMA, 300 ng/mL ionomycin, PMA plus ionomycin or 10 ng/mL
663 TNF α for 30 min. Cell lysates were subjected to Western blotting analysis.

664 C Jurkat cells were pretreated with 1 μ M Trametinib (MEK1/2 inhibitor), 500 nM
665 Bisindolylmaleimide VIII (BIMVIII) (PKC inhibitor), 1 μ M, 50 μ M SP6000125 (JNK
666 inhibitor), or 5 μ M SB203580 (p38 inhibitor), and subsequently treated with CD3 plus
667 CD28 (1 μ g/ μ L each). Western blotting analysis was performed as indicated.
668 Treatment with SB203580 gave non-specific results as it also regulated ERK
669 phosphorylation. Yellow symbol indicates CYLD Cter cleavage band.

670 D HEK293T cells were co-transfected with plasmids encoding for HOIP, SHARPIN or
671 HOIP plus SHARPIN together with ERK1-FLAG. Cells were lysed with TNT. Cell
672 lysates were immunoprecipitated using anti-FLAG M2 affinity gel and subjected to
673 Western blotting analysis.

674 E The LUBAC was pulled down by immunoprecipitation of Jurkat cell lysates using the
675 HOIL-1 antibody. *In vitro* kinase assay was performed by adding active ERK1, ERK2-
676 GST or ERK1 plus ERK2-GST recombinant proteins to HOIL-1 pulled down beads.

677 Western blotting analysis was performed for the indicated antibodies. White and
678 green arrows show SHARPIN and phosphorylated SHARPIN, respectively. Molecular
679 weight markers (M_r) are indicated.

680

681 **Figure EV2, relative to Figure 2. Characterization of SHARPIN knockout Cells**

682 A SHARPIN identified during mass spectrometry analysis (peptide rank1, peptide score
683 > 30, FDR<1% at PSM level). The protein score is the sum of the unique peptide
684 score calculated following $-10\log_{10}(p)$, where p is the absolute probability. Peptide
685 count and spectral count are indicated.

686 B Mass spectrometry analysis of immunoprecipitates prepared from Jurkat cell lysates
687 with antibodies to SHARPIN identified phosphorylation sites. The identified peptides
688 were validated with an FDR < 1% and a minimum score of 30. The peptide score
689 calculated in the Mascot search engine following $-10\log_{10}(p)$, where p is the absolute
690 probability. The position of the post-translational modification in the protein (PTM), the
691 number of peptides identified, the peptide score, the peptide sequence with the
692 modified amino acid in blue the localization confidence value of the post-translational
693 modification calculated in the Mascot search engine (ptm sites confidence) and the
694 number of spectra detected for each peptide (#PSM or peptide-spectrum match).

695 C Scheme showing the sgRNA target and PAM site on SHARPIN for the LentiCRISPR
696 vector containing SHARPIN sgRNA.

697 D Western blotting analysis of Jurkat single cell clones targeted with sgSHARPIN
698 LentiCRISPRv2 (knock down, KD or an empty vector (EV)).

699 E Jurkat *Sharpin* KO cells were complemented with an empty vector (EV), or with wild-
700 type (WT), S165A-, S312A- or S165A+S312A (2SA)-SHARPIN. Cells were stimulated
701 for 30 min with 20 ng/mL PMA plus 300 ng/mL ionomycin. Cell lysates were
702 subjected to Western blotting analysis. White and green symbols show SHARPIN and
703 phosphorylated SHARPIN, respectively. Molecular weight markers (M_r) are indicated.

704 F NF- κ B reporter luciferase assay of Parental Jurkat and Jurkat knockout (KO) cells
705 treated for 6 h with 20 ng/mL PMA plus 300 ng/mL ionomycin, CD3 plus CD28 (1
706 μ g/mL each) or 1 ng/mL TNF α (mean \pm SEM; * p <0.05, ** P <0.01 by one-way ANOVA,
707 $n=2$).

708 G Cell lysates from Jurkat, *Sharpin* KO Jurkat cells or cells reconstituted with EV, WT-,
709 S165A- or S165D-SHARPIN. Western blotting analysis was performed for the
710 indicated proteins.

711

712 **Figure EV3, relative to Figure 3.**

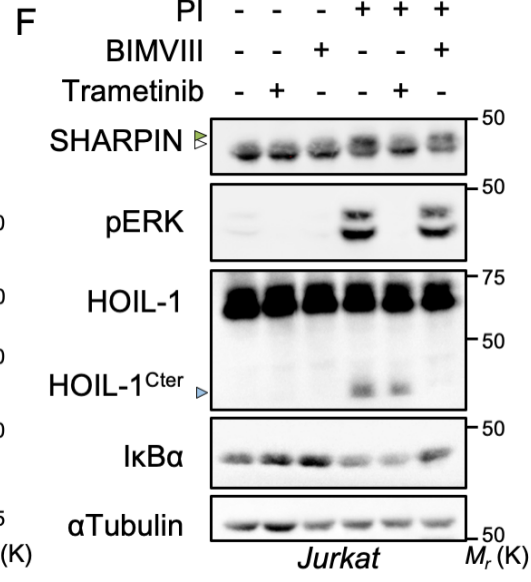
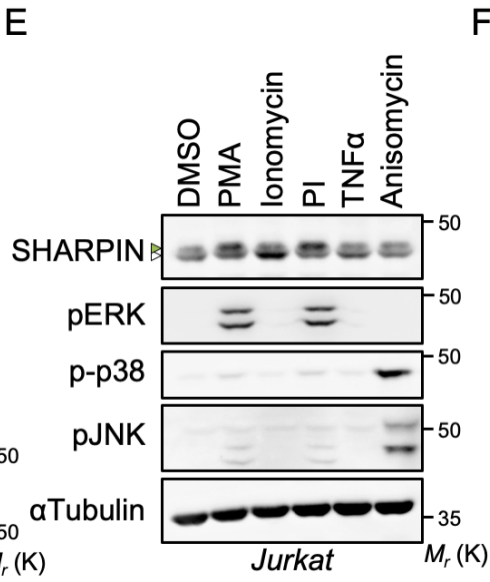
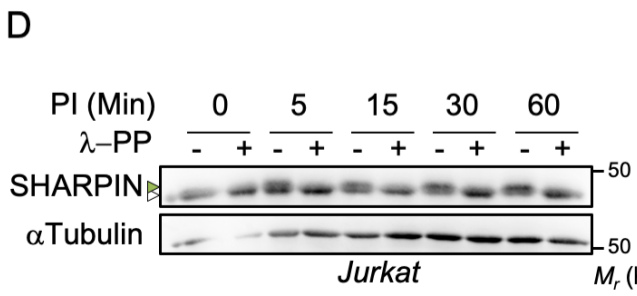
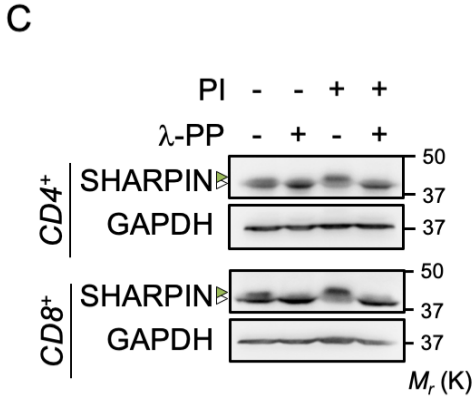
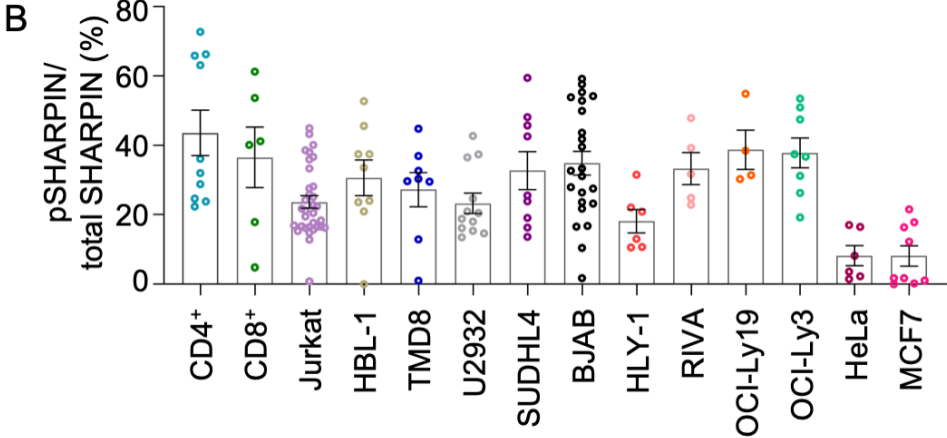
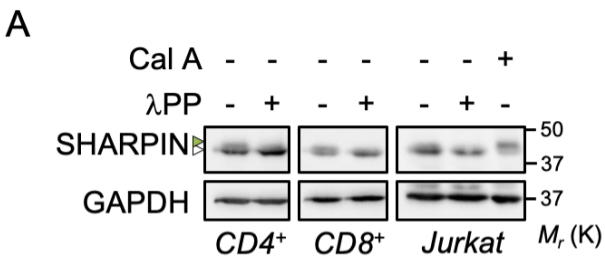
713 A-B Jurkat cells (A) or *Sharpin* knockout (KO) Jurkat cells, reconstituted with wild type
714 (WT-) or S165A-SHARPIN (B) were stimulated with 100 ng/mL TNF α -FLAG for the
715 indicated time points.). Cell lysates were immunoprecipitated using anti-FLAG M2
716 affinity gel (A-B). Beads were incubated with or without lambda phosphatase (λ PP)
717 (A) and subjected to Western blotting analysis (A-B). White and green symbols show
718 SHARPIN and phosphorylated SHARPIN, respectively. Molecular weight markers
719 (M_r) are indicated.

720 C-D Cell lysates of WT- and S165A-expressing Jurkat were treated with 10 ng/mL TNF α
721 (C) or with 20 ng/mL PMA plus 300 ng/mL ionomycin (PI) (D) as indicated and
722 subjected to Western blotting analysis.

723 E WT- or S165A-SHARPIN reconstituted Jurkat cells were stimulated with PI or TNF α
724 for 30 min as in (B-C) before performing TransAM NF- κ B activation assay for cREL
725 NF- κ B subunit. (mean \pm SEM; * p <0.05, ** P <0.01 by two-way ANOVA, $n=2$).

726 F-G List of RT² profiler PCR array of human NF- κ B signaling targets with an expression
727 fold change >2 in in cells expressing S165A-SHARPIN when compared to WT-
728 SHARPIN. Cells were treated during 4h with 20 ng/mL PMA plus 300 ng/mL
729 ionomycin (F) or 10 ng/mL TNF α (G). Fold regulation and p-value are listed.

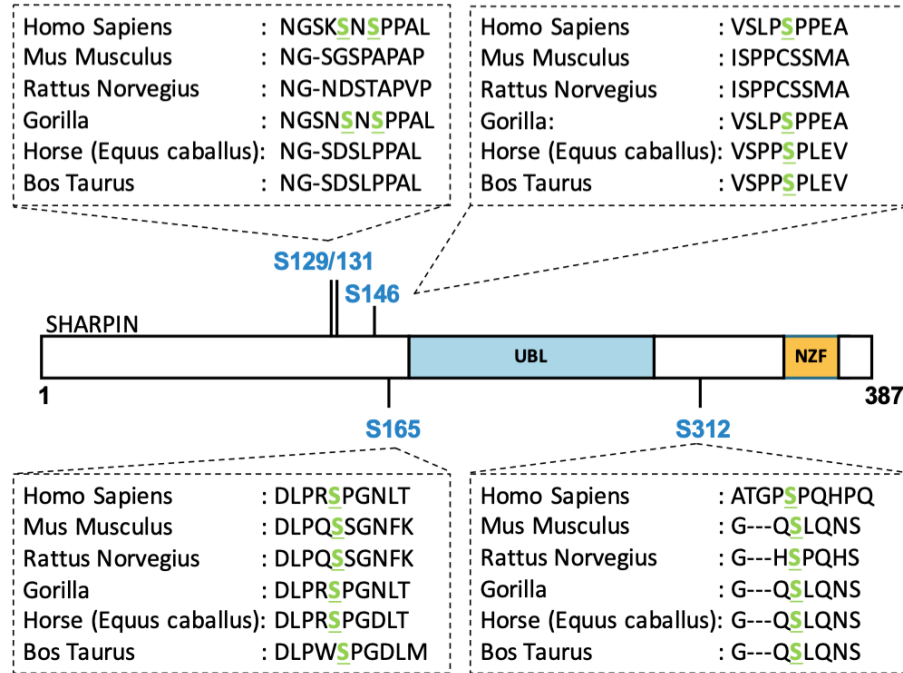
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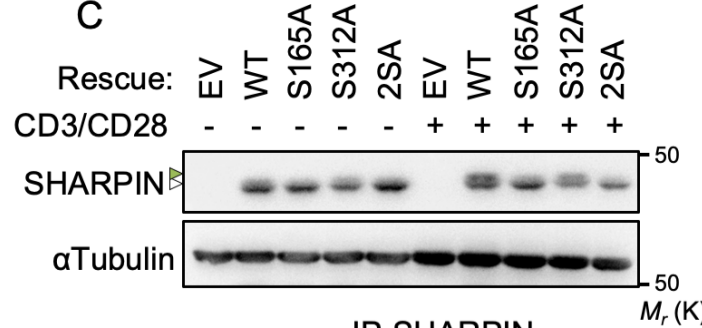
A

PTM Protein Position	Site Probability	Pep. No.	Sequence
Phospho S129	65,20%	1	SNSPPALGPEACPVS LPSPPEASTLK
		2	SNSPPALGPEACPVS LPSPPEASTLK
Phospho S131	50%	1	SNSPPALGPEACPVS LPSPPEASTLK
		2	SNSPPALGPEACPVS LPSPPEASTLK
Phospho S146	92.44%	3	SNSPPALGPEACPVS LPSPPEASTLK
		2	SNSPPALGPEACPVS LPSPPEASTLK
		2	SNSPPALGPEACPVS LPSPPEASTLK
Phospho S165	100%	4	SPGNLTER
Phospho S312	89.49%	5	EAPATGSPQHPQK

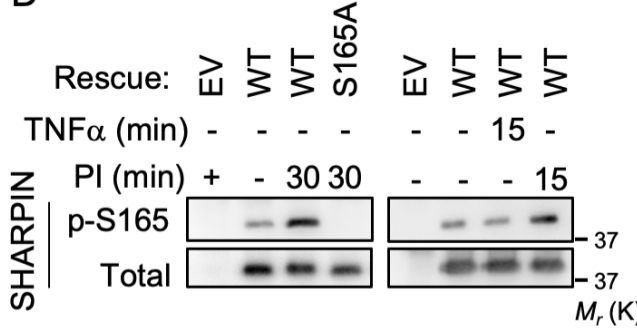
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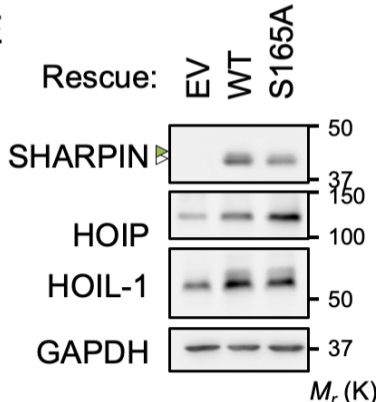
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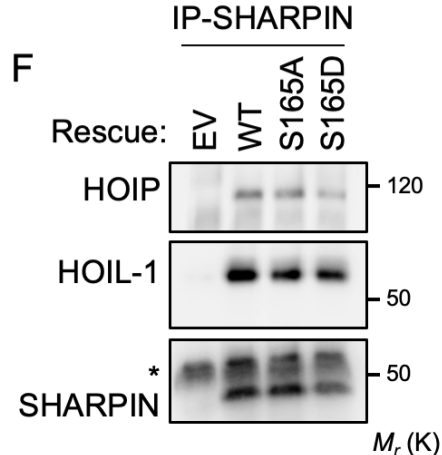
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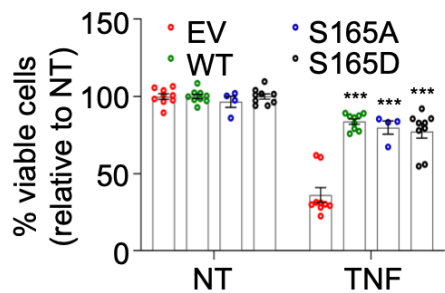
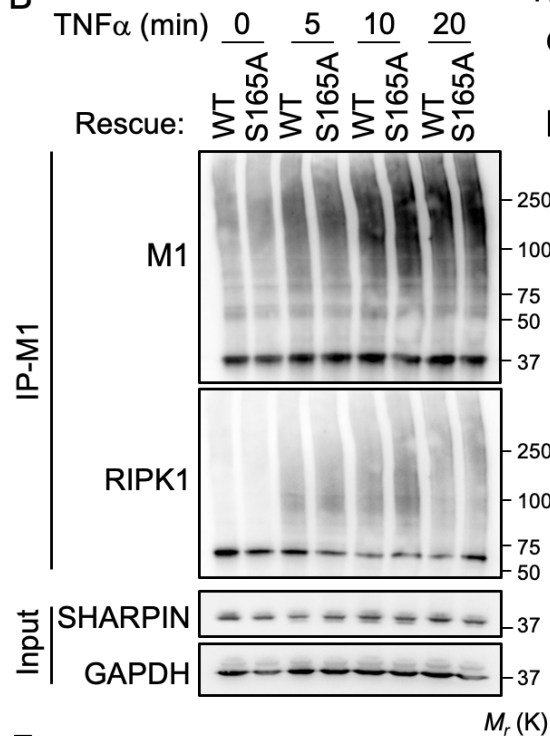
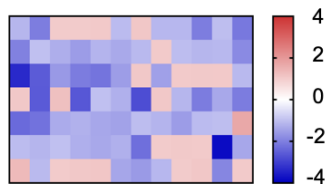
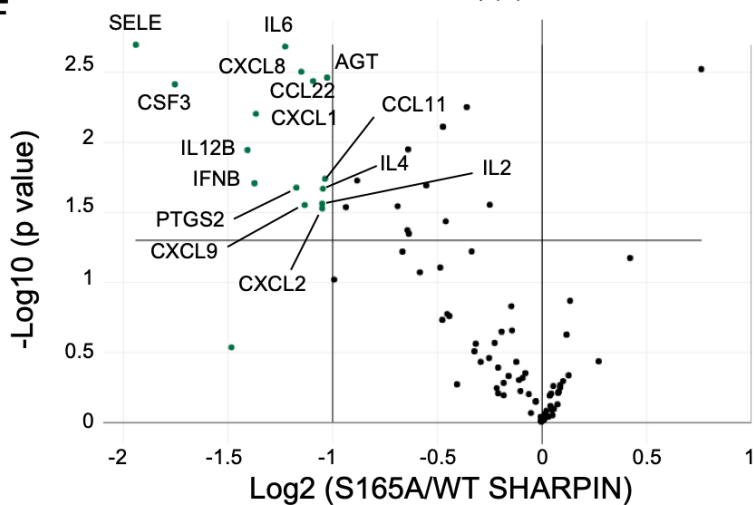


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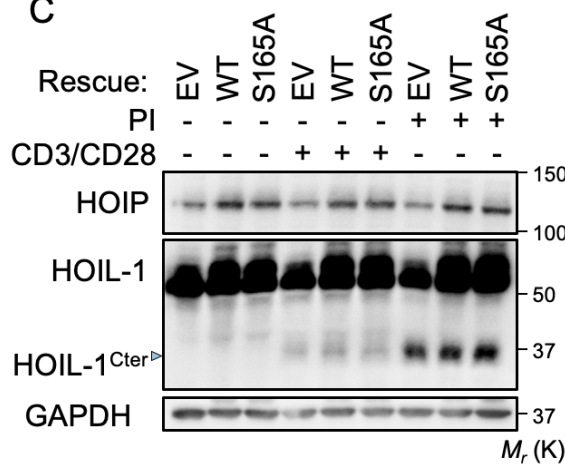
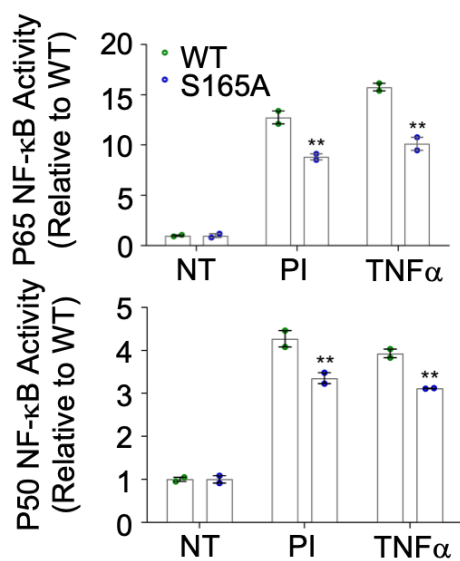
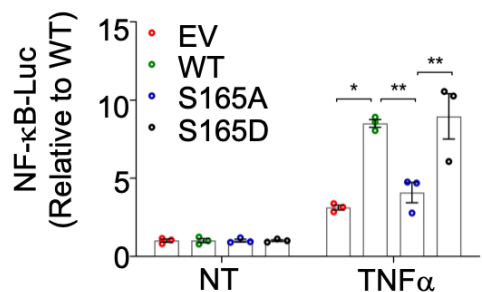
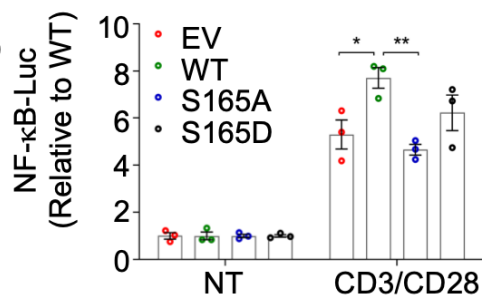
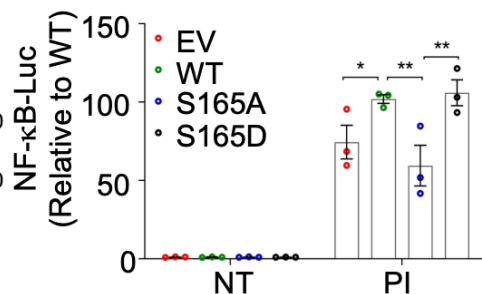
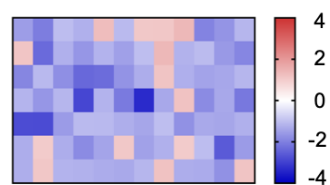
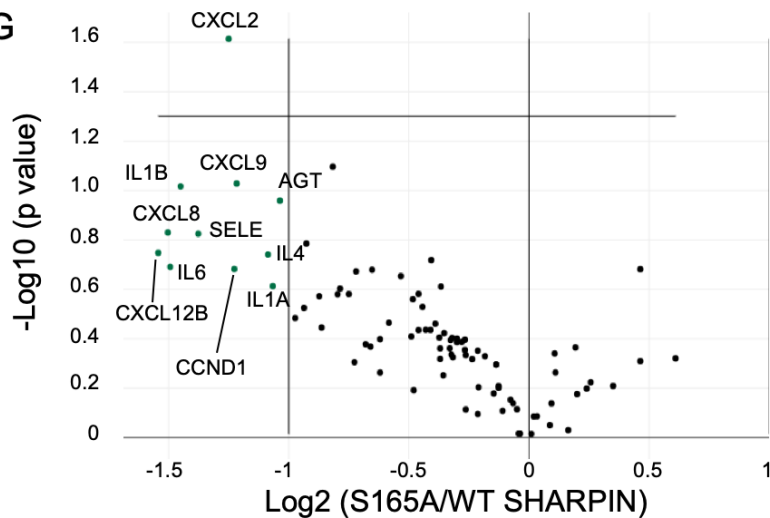


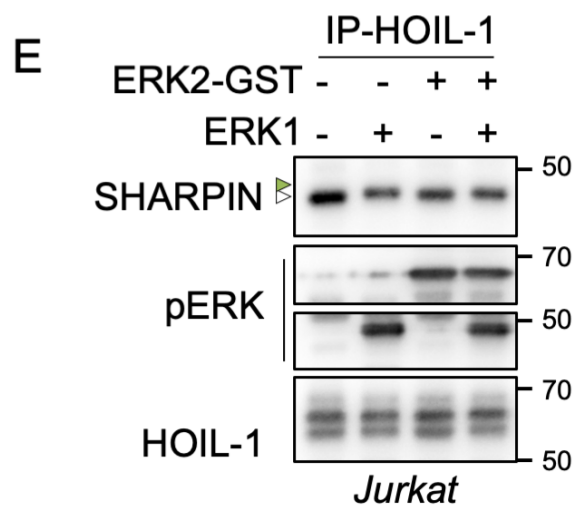
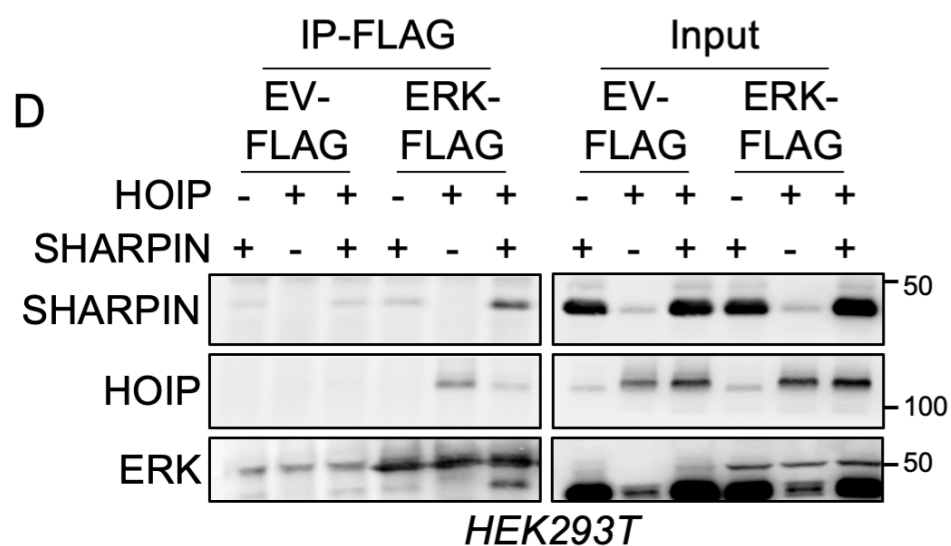
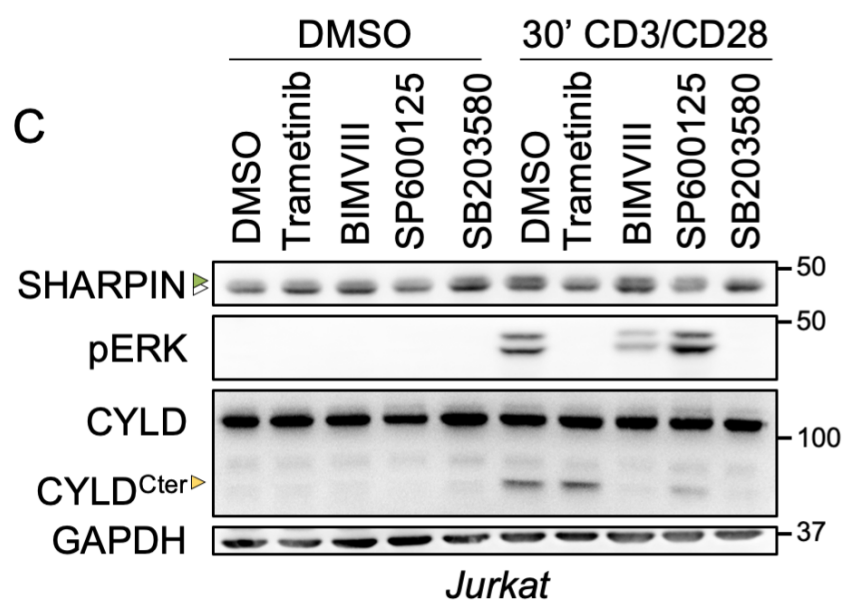
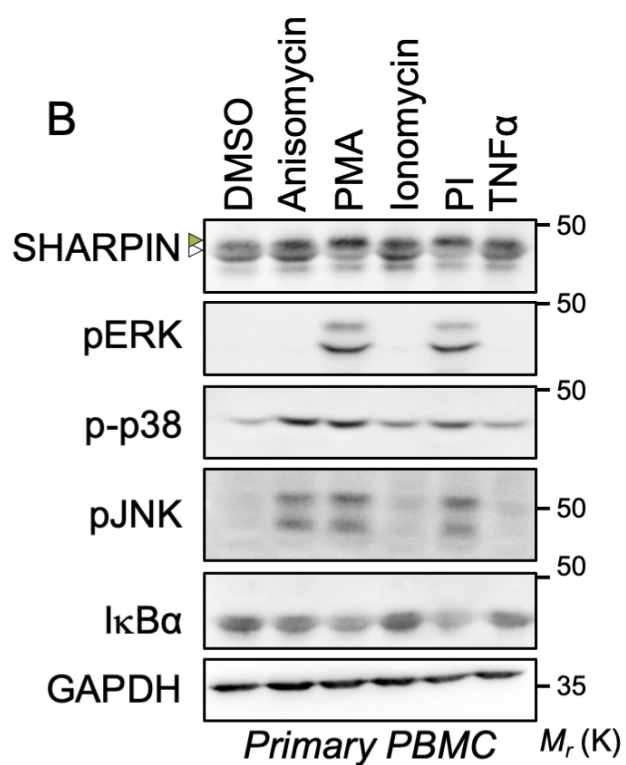
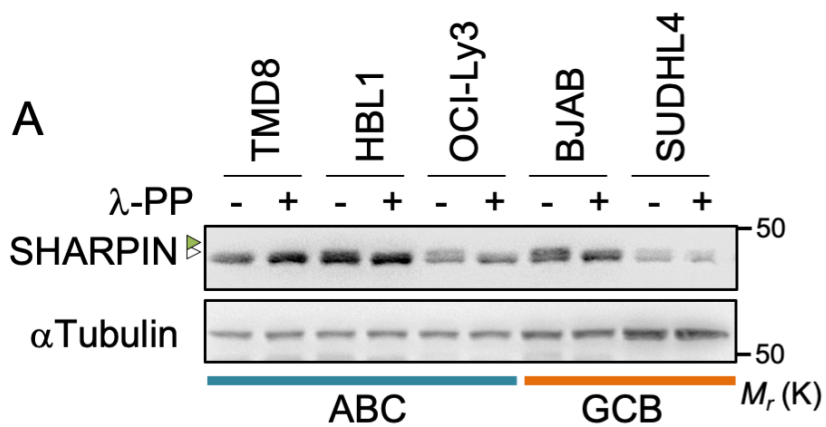
F



A**B****F**

PI

C**E****D****G**TNF α

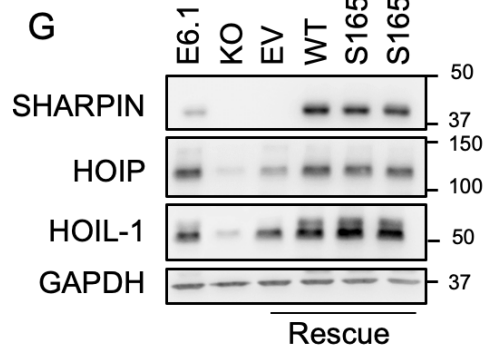
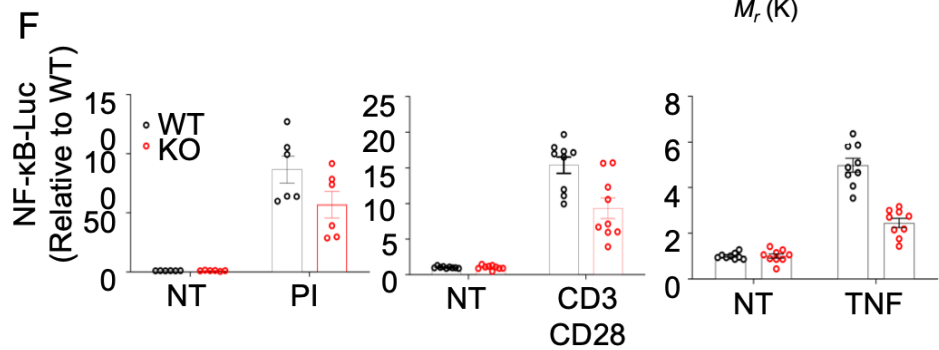
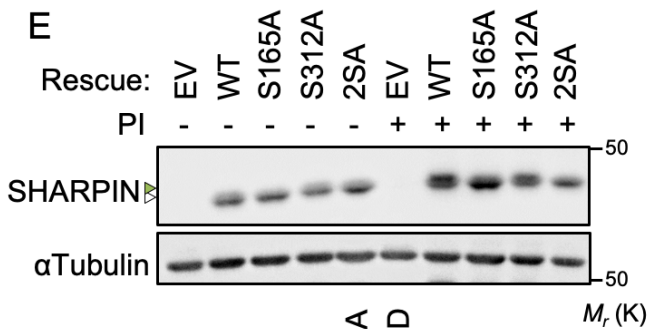
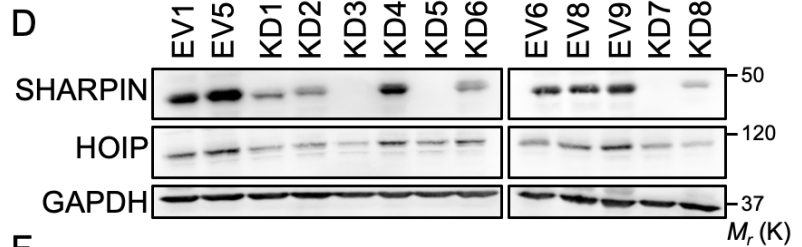
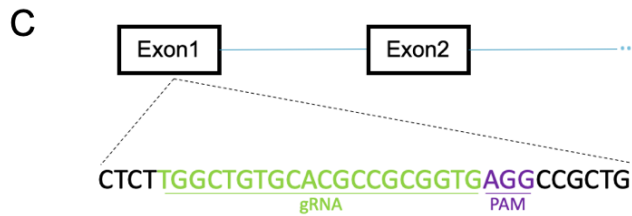


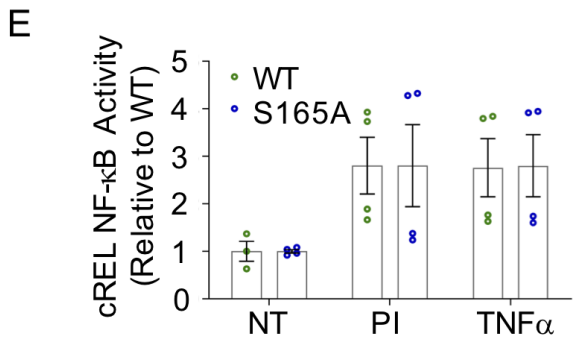
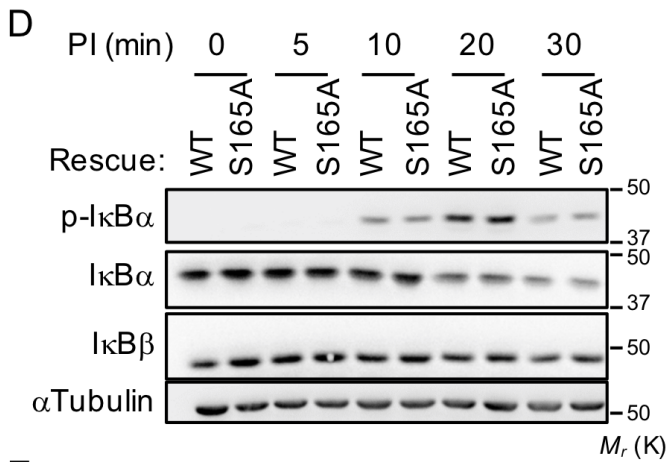
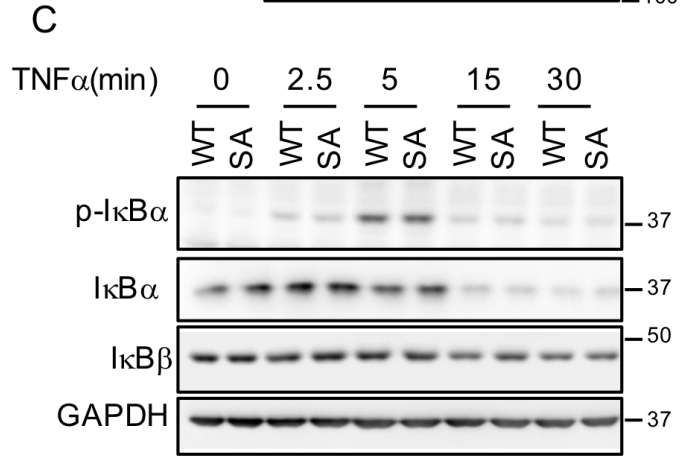
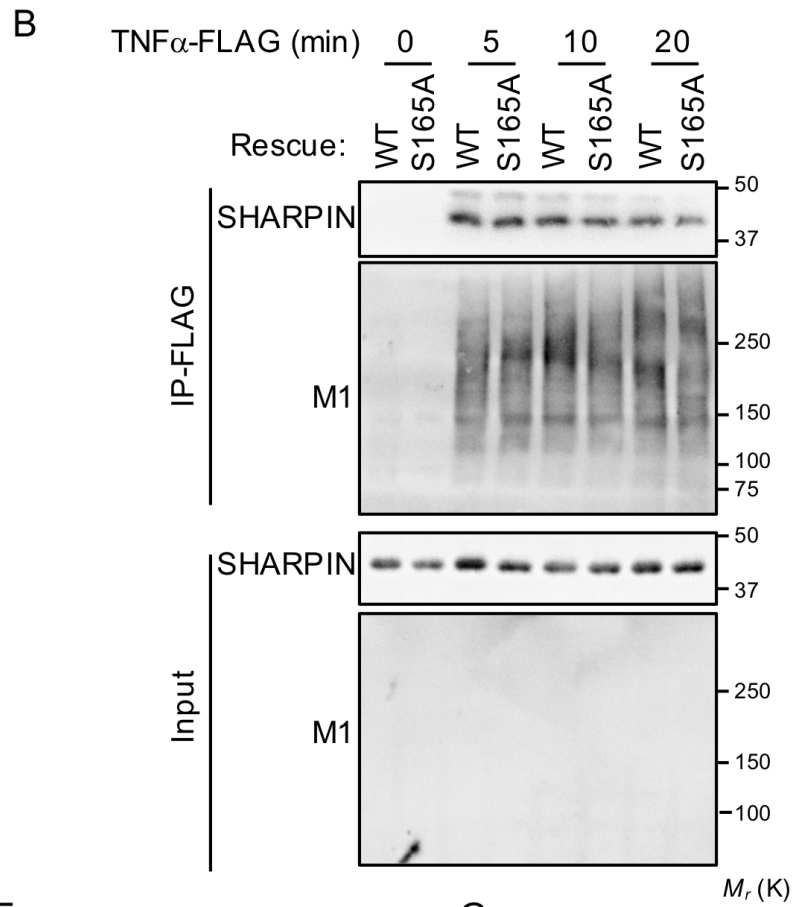
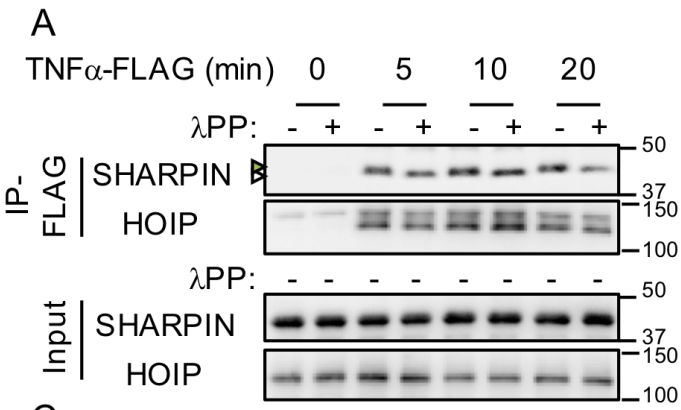
A

Protein set	Description	Score	Peptides	Spectral Count
Sp/Q9H0F6/SHRPN_HUMAN	Sharpin OS=Homo sapiens (Human) OX=9606 GN=SHARPIN PE=1 SV=1	1048.03	20	61

B

PTM Protein Position	Pep. No.	Score	Sequence	PTM Sites Confidence	#PSM
Phospho S129	1	49.06	SNSPPALGPEACPVSLSPEASTLK	Phospho (S1) = 50%	3
	2	61.81	SNSPPALGPEACPVSLSPEASTLK	Phospho (S1) = 50% Phospho (S18) = 92%	2
Phospho S131	1	49.06	SNSPPALGPEACPVSLSPEASTLK	Phospho (S3) = 50%	3
	2	61.81	SNSPPALGPEACPVSLSPEASTLK	Phospho (S3) = 50% Phospho (S18) = 92%	1
Phospho S146	3	44.59	SNSPPALGPEACPVSLSPEASTLK	Phospho (S18) = 91%	1
	2	61.81	SNSPPALGPEACPVSLSPEASTLK	Phospho (S1) = 50% Phospho (S18) = 92%	2
	2	61.81	SNSPPALGPEACPVSLSPEASTLK	Phospho (S3) = 50% Phospho (S18) = 92%	1
Phospho S165	4	41.63	SPGNLTER	Phospho (S1) = 100%	2
Phospho S312	5	38.87	EAPATGSPQHPQK	Phospho (S8) = 89%	1





F

Gene Symbol	Fold regulation	P value
SELE	-3.84	0.002010
CSF3	-3.37	0.003848
IL12B	-2.65	0.011335
IFNB1	-2.59	0.019553
CXCL1	-2.58	0.006250
IL6	-2.34	0.002069
PTGS2	-2.26	0.021027
CXCL8	-2.22	0.003132
CXCL9	-2.19	0.027996
CCL22	-2.13	0.003650
CXCL2	-2.07	0.029537
IL2	-2.07	0.027333
IL4	-2.07	0.021390
CCL11	-2.05	0.018191
AGT	-2.04	0.003451

G

Gene Symbol	Fold regulation	P value
CXCL2	-2.38	0.024300
IL12B	-2.92	0.178692
CXCL8	-2.83	0.147931
IL6	-2.82	0.203720
IL1B	-2.73	0.096160
SELE	-2.60	0.149616
CCND1	-2.34	0.207953
CXCL9	-2.32	0.093671
IL4	-2.12	0.181482
IL1A	-2.09	0.243840
AGT	-2.05	0.109868