

# Serine 165 Phosphorylation of SHARPIN regulates the Activation of NF- $\kappa B$

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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-kB 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- $\kappa$ B in response to TNF $\alpha$  and T-cell receptor engagement. 36 These results identify a new layer of regulation of the LUBAC, and unveil new strategies to modulate its action. 37

# 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 C3HC4type zinc finger-containing protein 1, also called RBCK1, HOIL-1L) [1-3]. This unique 43 44 complex catalyzes the formation and attachments of atypical linear ubiquitin chains on substrates, thereby modifying their fate. The LUBAC acts as a linchpin by transducing 45 signals from most immunoreceptors to NF- $\kappa$ B, and therefore emerges as a key regulator of 46 47 innate and adaptive immunity [1–13]. For instance, the binding of tumor necrosis factor  $\alpha$ (TNFa) to its cognate receptor TNFR1 (TNF receptor 1) drives the recruitment of the LUBAC 48 into the so-called complex I. By decorating the key protein kinase RIPK1 (receptor-interacting 49 50 protein 1 kinase) with linear ubiquitin chains, the LUBAC stabilizes and favors downstream 51 NF-κB signaling [1–3,7]. Mice deficient in SHARPIN, HOIP or HOIL-1 are hallmarked by an 52 exacerbated TNFa 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 dermatitis HOIP and HOIL-1 deficiency is embryonically lethal [1-3,14-16]. The LUBAC also 56 57 functions downstream of antigen receptors to ensure the optimal activation of NF-kB, and 58 this signaling pathway is pirated in the activated B cell like subtype of diffuse large B-cell lymphomas (ABC DLBCL) [17–20]. Accordingly, the targeting of the LUBAC was shown to be 59 60 toxic in ABC DLBCL, unveiling a contribution of this complex to lymphomagenesis [19–22].

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

than other substrates [25]. Linear auto-ubiquitination of the LUBAC can be removed by the 66 67 OTU deubiquitinase with linear linkage specificity (OTULIN) [23,24]. In addition, HOIP is 68 processed upon TNFα- and TRAIL-induced apoptosis by caspases, with cleaved fragments 69 displaying reduced NF-κB activation capabilities [26,27]. HOIP is also phosphorylated by 70 mammalian ste20-like kinase 1 (MST1) in response to TNFa, and this modulates its E3 71 ligase activity, thereby attenuating NF- $\kappa$ 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-κB [29-31]. 74 Lastly, SHARPIN is decorated with lysine (K) 63 ubiguitin 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-κB 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-kB response to both T-cell receptor engagement and TNFa 82 stimulation.

83

# **Results and discussion**

#### 85 SHARPIN is a phosphoprotein

Western blotting analysis of SHARPIN, in human primary CD4<sup>+</sup> and CD8<sup>+</sup> cells, Jurkat cells and DLBCL cell lines, revealed that SHARPIN resolves as a doublet (Fig. 1A, EV1A). The treatment of cell lysates with lambda phosphatase, which removes phosphate groups from serine, threonine and tyrosine, effectively chased away the slow migration specie of SHARPIN, suggesting phosphorylation (Fig.1A, EV1A). Conversely, incubating cells with the phosphatase inhibitor Calyculin A resulted in an increase intensity of the slow migration specie of SHARPIN, reinforcing the idea that SHARPIN is indeed phosphorylated (Fig. 1A).
Quantification of constitutive SHARPIN phosphorylation in numerous cell lines, revealed that
phospho-SHARPIN exists at various levels within different cell types, with lymphoid cells
having a relatively high SHARPIN phosphorylation (Fig. 1B). Hence, a fraction of SHARPIN
is constitutively phosphorylated.

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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 TNFa 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 ionomycin or CD3 plus CD28 stimulation, brought SHARPIN phosphorylation back to basal 108 109 levels, but did not prevent this constitutive mark (Fig. 1F, EV1C). By contrast, inhibition of the 110 NF- $\kappa$ B pathway with the PKC inhibitor BisindolyImaleimide 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

phosphorylation of SHARPIN, one, yet unidentified, that phosphorylates SHARPIN in resting
conditions, and ERK1/2, which phosphorylates SHARPIN upon antigen receptor
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 putative SHARPIN phosphorylation sites (S165A, S312A, S165A+S312A (2SA)). S165 135 136 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 TNFα stimulation (Fig. 2D).

143

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

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

151

#### 152 SHARPIN phosphorylation contributes to the optimal activation of NF-κB

153 The LUBAC is recruited to the TNFR1 complex I upon TNFα stimulation, where it is crucial 154 for stabilizing the complex and subsequent NF-κB 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 IkB kinase (IKK) 157 complex, consisting of IKK $\alpha$ , IKK $\beta$  and NEMO, with NEMO itself being modified with M1-158 chains. When activated, the IKK complex phosphorylates NF-KB inhibitors IKBs leading to 159 their degradation. This liberates NF-kB 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 TNFa stimulation. As previously shown [1-3], TNFa stimulation of Sharpin knockout cells 163 164 reconstituted with an empty vector effectively reduced cell viability, while reconstitution with WT-, S165A-, or S165D-SHARPIN resulted in a similar protective effect against TNFα 165 166 (Fig.3A). Even though phosphorylated SHARPIN is detected at the TNFR1, upon TNF $\alpha$ -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 $\alpha$  stimulation did not drive any significant changes in total M1 ubiguitination 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,  $I\kappa B\alpha$  phosphorylation was unchanged in cells stimulated with 178 TNF $\alpha$  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-κB (Fig. 3D-G). NF-κB family members control the transcription of cytokines, and 184 regulate cellular differentiation, survival and proliferation [36]. The NF-κB family is composed 185 of five related transcription factors in mammals: p50, p52, p65 (also called ReIA), c-ReI and 186 RelB [37]. As expected, the transcription activity of NF- $\kappa$ B, as measured by a NF- $\kappa$ B reporter 187 luciferase assay, was reduced in *Sharpin* knockout cells, reconstituted with an empty vector, 188 stimulated through antigen receptor engagement or  $TNF\alpha$ , when compared to cells 189 expressing WT-SHARPIN (Fig. 3D). Interestingly, NF-κB 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-kB subunits p65 and p50 upon antigen receptor engagement or TNFa 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-kB signaling targets showed a significant diminished expression of numerous cytokines (CSF3, IL12B, IFNBA, IL6, CXCL9, CCL22, IL2 and IL4) 196 and chemokines (CXLCL1, CXCL2, CXCL8, CCL11) in S165A-SHARPIN expressing cells 197 198 treated with PMA and ionomycin (Fig. 3F, Fig. EV3F). Similar results were found when cells 199 were stimulated with TNF $\alpha$ , albeit only the chemokine receptor CXCR2 reached statistical 200 significance there is a trend towards reduced expression of other NF-kB signaling targets 201 (Fig. 3G, EV3G). It should be mentioned that compared to PMA plus ionomycin stimulation, 202 induction of NF- $\kappa$ B target genes by TNF $\alpha$  was weaker, which may also explain why 203 differences upon TNF $\alpha$  stimulation are less striking. Nevertheless, we established that the 204 phosphorylation of SHARPIN on S165 is participates in NF- $\kappa$ B upon antigen receptor 205 engagement and TNF $\alpha$  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 provide evidence that SHARPIN is further phosphorylated on the Serine 165 by ERK upon 209 210 antigen receptor engagement, but not in response to TNF $\alpha$  (Fig. 1C). Yet, the constitutive 211 phosphorylation of SHARPIN is pivotal for the optimal activation of NF-κB in response to 212 antigen receptor engagement and TNFR ligation. This apparent dichotomy militates against a 213 role for the inducible phosphorylation in NF- $\kappa$ B activation. It is therefore tempting to speculate that ERK-mediated phosphorylation of SHARPIN plays an independent function. 214 215 Beside its crucial role in NF-kB signaling, a growing body of literature suggests that SHARPIN also acts as an inhibitor of the integrin adhesion receptors [38-40]. Pouwels et al. 216 217 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

speculate that different kinases could target different LUBAC complexes, and consequentlyexert selective functions.

229

230 Although necessary for complete NF- $\kappa$ B activity upon antigen receptor engagement and 231 TNFα stimulation, how SHARPIN phosphorylation regulates the LUBAC functions and NF-κB 232 signaling is unclear. The basal S165 SHARPIN phosphorylation appeared dispensable for 233 TNFα-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-κB transcription activity. Nevertheless, this may also open up new avenues for therapeutic prospects of aggressive lymphoma, such as ABC DLBCL, 236 237 for which the LUBAC and NF-κB 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 $\alpha$ -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- $\kappa$ 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

Jurkat E6.1 T lymphocytes, Hela, HEK293T and MCF7 cells were purchased from American Type Culture Collection (ATCC). U2932, SUDHL4, BJAB, RIVA, OCI-LY3, and OCI-LY19 cells were acquired from DSMZ. HBL1, HLY1 and TMD8 cells were kindly provided by Martin Dyer, Pierre Brousset, and Daniel Krappmann, respectively. Blood was obtained from healthy donors (Etablissement Français du Sang). Peripheral blood mononuclear cells (PBMC) were acquired by Ficoll density gradient. Primary CD4<sup>+</sup> and CD8<sup>+</sup> T cells were subsequentially isolated from PBMC using REAlease CD4 and CD8 microbeads kits, from
Miltenyi as manufacturer's instructions. Cell viability was assessed using CellTiter-Glo
following manufacturer's instructions (Promega) following stimulation with 10 ng/mL TNFα
(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 µg/mL CD3 261 plus 1 µg/mL CD28 (Becton Dickinson Biosciences), 12.5 mg/mL anisomycin (Merck), 10 ng/mL TNFα (R&D systems) or TNFα-Flag (Enzo Life Sciences). To ensure inhibition of 262 263 certain signaling pathways Jurkat E6.1, PBMC or primary T cells were pre-treated during 1h 264 with 1 µM Trametinib (Selleckchem), 500 nM BisindolyImaleimide VIII (BIMVIII, Enzo), 50 µM 265 SP6000125 (Cell Signaling), or 5 µM SB203580 (Selleckchem). Cells were washed with icecold PBS and subsequently lysed using two possible lysis buffers, as indicated throughout 266 267 the manuscript. (1) Cells pellets were incubated with 188 µl 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 Na<sub>3</sub>VO<sub>4</sub>) for 5 min 269 on ice. 12 µl 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-HCI (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 µg of protein was incubated with 600 Units of lambda phosphatase, 1x NEB buffer 278 for Protein Metallophosphatase and 1mM MnCl<sub>2</sub> (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  $I\kappa B\beta$  (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), IkBa (#9242), phospho-287 IκBα (#9246), phospho-p38 (#9215), phospho-JNK (#9255) and RIP1 (#3493) were acquired from Cell Signaling Technologies. Anti-linear Ubiquitin (MABS199) was procured from 288 289 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

HOIL-1. Beads were washed in kinase buffer (5 mM MOPS pH 7.2, 5 mM MgCl<sub>2</sub>, 1 mM
EGTA, 0.4 mM EDTA, 0.05 mM DTT). Beads were subsequently incubated with kinase
buffer, 2mM ATP, 350 ng/mL active untagged ERK1 (Merck), 350 ng/mL active ERK2-GST
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 37°C, and subsequently alkylated by 135 mM lodoacetamide for 15 min at room 319 320 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 nanochromatography (nanoLC) nanoElute coupled with a TimsTOF Pro mass spectrometer 325 326 (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 SHARPIN guide RNA (5' CACCGTGGCTGTGCACGCCGCGGTG 3'), as described before 344 345 (Sanjana et al., 2014; Shalem et al., 2014). LentiCRISPRv2, together with the packaging vectors PAX2 and VSV-G, were transfected in HEK293T cells using a standard calcium 346 347 phosphate protocol, as previously published [54]. Supernatant, containing the lentiviral particles, was collected after 48 h, and used to infect 10<sup>6</sup> Jurkat cells in presence of 8 µg/mL 348 Polybrene (Santa Cruz Biotechnology). Jurkat cells expressing the LentiCRISPRv2 vector 349 350 were selected by adding 1µg/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 using the pCMH-MSCV-EF1a-puroCopGFP vector containing wild type (WT) SHARPIN, to 354 355 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).

#### 359 NF-кB assays

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

370

#### 371 Statistical analysis

372 Statistical analysis, comparing multiple groups was performed using two-way ANOVA on rank test with Tuckey's post hoc test in GraphPad Prism 7 software. TransAM NF-κB 373 374 activation assays were analyzed using a 2-way ANOVA test with a Sidak correction for 375 multiple analysis. RT<sup>2</sup> profiler PCR array was performed using the online software provided 376 by the manufacturer (https://dataanalysis2.giagen.com/pcr). In short, the p-values were calculated based on a student t-test of the replicate  $2^{-}(-DC_{T})$  values for each gene in the 377 378 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.

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# 403 **Conflict of interest**

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

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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<sup>+</sup> or CD8<sup>+</sup> T lymphocytes and Jurkat cells 574 were treated with lambda phosphatase ( $\lambda$ 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<sup>+</sup> cells, primary human T CD8<sup>+</sup> 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<sup>+</sup> T (E) and CD8<sup>+</sup> 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  $\lambda$ 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 TNFα or 12.5 μg/mL Anisomycin, for 30 min. Cell lysates 588 were subjected to Western blotting analysis.
- 589 F Jurkat cells we pretreated for 1h with 1 μM Trametinib or 500 nM BIMVIII and 590 subsequently stimulated and lysed as in (E). Blue arrowhead indicates HOIL-1 Cter.
- 591

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

594 A Mass spectrometry analysis of immunoprecipitates prepared from Jurkat cell lysates 595 with antibodies to SHARPIN identified phosphorylation sites. The identified peptides were validated with an FDR < 1% and a minimum score of 30. The peptide score calculated in the Mascot search engine following  $-10\log_{10}(p)$ , where p is the absolute probability. The position of the post-translational modification in the protein (PTM), the modification site probability calculated in Proline (site probability) the number of peptides identified, the peptide sequence with the identified amino acid in blue the localization and other amino acid modifications in green.

B Sequence alignment of the amino acids surrounding the serine (S) 129, S131, S146,
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  $\mu$ 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<sub>r</sub>*) are indicated.

D Sharpin knockout Jurkat cells reconstituted with EV, WT- or S165A-SHARPIN were
 treated with PI as in (C), or with 10 ng/mL TNFα. Cells lysates were
 Immunoprecipitated (IP) with antibodies specific to SHARPIN and subjected to
 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.

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

621

Figure 3: Serine 165 Phosphorylation of SHARPIN is crucial for the optimal Activation
of NF-κB.

624ASharpin knockout Jurkat cells expressing an empty vector (EV) or wild type (WT-),625S165A- or S165D-SHARPIN were stimulated with 10 ng/mL TNFα for 24 h. Cell626viability was assessed with CellTiter glo (mean  $\pm$  SEM; \*\*\*P<0.001 by two-way</td>627ANOVA).

B WT- and S165A-expressing Jurkat cells were stimulated with 10 ng/mL TNFα as indicated. Cell lysates were subjected to immunoprecipitation (IP) of linear ubiquitin (M1) prior to Western blotting analysis with the specified antibodies. Molecular weight 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 μg/mL each) for 30 min. Cell lysates were subjected to western
635 blotting analysis. Blue symbol indicates HOIL-1 Cter cleavage band.

636DNF-κB reporter luciferase assay in the indicated Jurkat cells treated for 6 h with 20637ng/mL PMA plus 300 ng/mL ionomycin (PI), 1 µg/mL anti-CD3 plus 1 µg/mL anti-638CD28, or with 1 ng/mL TNFα. Graph displays 1 out of 3 experiments done in triplicate639(mean ± SEM; \*p<0.05, \*\*P<0.01 by two-way ANOVA).</td>

640 E WT- or S165A-SHARPIN reconstituted Jurkat cells were stimulated with PI or TNFα 641 for 30 min as in (D) before performing TransAM NF- $\kappa$ B activation assay for p65 and 642 p50 NF- $\kappa$ B subunits. The presented Graph show 1 out of 3 experiment performed in 643 duplicate (mean ± SEM; \*p<0.05, \*\*P<0.01 by two-way ANOVA).

F-G Volcano plot or  $RT^2$  profiler PCR array of human NF-κB signaling targets for cells stimulated for 4 h with PI (F) or TNFα (H). (G) Genes indicated on the volcano plot are significantly down regulated with a fold change > 2 in cells expressing S165A-SHARPIN when compared to WT-SHARPIN. On (G), CXCR2 is significantly down regulated with a fold change >2, while other genes indicated are borderline significant in S165A-SHARPIN expressing cells, compared to WT-SHARPIN. (F-G) Heatmap in

- the below volcano plot gives an overall view of the gene expression in the RT<sup>2</sup> profiler
  PCR array.
- 652

# 653 Expanded View Figure legends

# Figure EV1, relative to Figure 1 SHARPIN Phosphorylation by ERK upon Antigen 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 ( $\lambda$ 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<sub>r</sub>*) are indicated.
- B Primary peripheral blood mononuclear cells (PBMC) were stimulated with 12.5 μg/mL
   Anisomycin, 20 ng/mL PMA, 300 ng/mL ionomycin, PMA plus ionomycin or 10 ng/mL
   TNFα for 30 min. Cell lysates were subjected to Western blotting analysis.
- Ge4 C Jurkat cells were pretreated with 1 μM Trametinib (MEK1/2 inhibitor), 500 nM
  BisindolyImaleimide VIII (BIMVIII) (PKC inhibitor), 1 μM, 50 μM SP6000125 (JNK
  inhibitor), or 5 μM SB203580 (p38 inhibitor), and subsequently treated with CD3 plus
  CD28 (1μg/μL each). Western blotting analysis was performed as indicated.
  Treatment with SB203580 gave non-specific results as it also regulated ERK
  phosphorylation. Yellow symbol indicates CYLD Cter cleavage band.
- D HEK293T cells were co-transfected with plasmids encoding for HOIP, SHARPIN or
  HOIP plus SHARPIN together with ERK1-FLAG. Cells were lysed with TNT. Cell
  lysates were immunoprecipitated using anti-FLAG M2 affinity gel and subjected to
  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, ERK2676 GST or ERK1 plus ERK2-GST recombinant proteins to HOIL-1 pulled down beads.

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- 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<sub>r</sub>) 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 В Mass spectrometry analysis of immunoprecipitates prepared from Jurkat cell lysates 687 with antibodies to SHARPIN identified phosphorylation sites. The identified peptides were validated with an FDR < 1% and a minimum score of 30. The peptide score 688 689 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 LentCRISPRv2 (knock down, KD or an empty vector (EV).

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

F NF- $\kappa$ B reporter luciferase assay of Parental Jurkat and Jurkat knockout (KO) cells treated for 6 h with 20 ng/mL PMA plus 300 ng/mL ionomycin, CD3 plus CD28 (1 µg/mL each) or 1 ng/mL TNFα (mean ± SEM; \*p<0.05, \*\*P<0.01 by one-way ANOVA, n=2).

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

711

712 Figure EV3, relative to Figure 3.

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

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

723EWT- or S165A-SHARPIN reconstituted Jurkat cells were stimulated with PI or TNFα724for 30 min as in (B-C) before performing TransAM NF- $\kappa$ B activation assay for cREL725NF- $\kappa$ B subunit. (mean ± SEM; \*p<0.05, \*\*P<0.01 by two-way ANOVA, n=2).</td>

F-G List of  $RT^2$  profiler PCR array of human NF-κB signaling targets with an expression fold change >2 in in cells expressing S165A-SHARPIN when compared to WT-SHARPIN. Cells were treated during 4h with 20 ng/mL PMA plus 300 ng/mL ionomycin (F) or 10 ng/mL TNFα (G). Fold regulation and p-value are listed.



				C << <<				
PTM Protein Position	Site Probability	Pep. No.	Sequence	Rescue: 2342 2342 2342 2342 2342 2342 2342 2342				
Phospho	65,20%	1	SNSPPALGPEACPVS LPSPPEASTLK	CD3/CD28 + + + + + + SHARPIN <sup>B</sup>				
S129		2	SNSPPALGPEACPVS LPSPPEASTLK	αTubulin				
Phospho	50%	1	SN <mark>S</mark> PPALGPEACPVS LPSPPEASTLK	D $\frac{\text{IP-SHARPIN}}{1}$				
S131		2	SN <mark>S</mark> PPALGPEACPVS LPSPPEASTLK	Rescue: A A A A A A A A A A A A A A A A A A A				
	92.44%	3	SNSPPALGPEACPVS LP <mark>S</mark> PPEASTLK	TNF $\alpha$ (min) 15 -				
Phospho S146		2	SNSPPALGPEACPVS LP <mark>S</mark> PPEASTLK					
		2	SNSPPALGPEACPVS LP <mark>S</mark> PPEASTLK	Total				
Phospho S165	100%	4	SPGNLTER					
Phospho 89.49% 5			EAPATGP <mark>S</mark> PQHPQK					
Homo Sapiens	: NGSK <mark>S</mark> N <mark>S</mark> PP	AL Hon	no Sapiens : VSLP <u>S</u> PP	HOIP 100				
Mus Musculus : NG-SGSPAPAP Rattus Norvegius : NG-NDSTAPVP			s Musculus : ISPPCSSN tus Norvegius : ISPPCSSN	HOIL-1				
Gorilla	: NGSN <u>S</u> NSPP	AL Gor	illa: : VSLP <u>S</u> PPI					
Horse (Equus caballus):       NG-SDSLPPAL       Horse (Equus caballus):       VSPPSPLEV       GAPDH       57         Bos Taurus       :       NG-SDSLPPAL       Bos Taurus       :       VSPPSPLEV       M_ (K								
•======================================		· ( ***==:						
	S129/131	,,, ,		IP-SHARPIN				
1				$\begin{array}{c} \blacksquare \\ 387 \\ \end{array} \qquad \qquad$				
	S16	5 	<u>S312</u>	HOIP 120				
Homo Sapiens Mus Musculus	: DLPR <mark>S</mark> PGNLT	Hon	no Sapiens : ATGP <u>S</u> PC					
Rattus Norvegius	: DLPQSSGNFK	Rat	tus Norvegius : GHSPC					
Gorilla Horse (Equus caball	: DLPR <u>S</u> PGNLT us): DLPRSPGDLT	Gor	illa : GQ <mark>S</mark> LC se (Equus caballus): GQSLC					
Bos Taurus	: DLPWSPGDL	M Bos	Taurus : GQ <mark>S</mark> LC	SHARPIN				

Α

В

. *M<sub>r</sub>* (К)





А	A Protein set			Description			Score	Peptie	des	Spectral Count		
	Sp/Q9H0F6/SHR	PN_HUM	AN	Sharpin OS=H GN=	harpin OS=Homo sapiens (Human) OX=9606 GN=SHARPIN PE=1 SV=1				20		61	
В	PTM Protein Position	Pep. No.	Score		Sequence			PTM Sites Confidence		#PSM		
	1 49.0			SNSPPAL	GPEACPVS	LPSPPEAST	LK F	Phospho (S1)	= 50%		3	Ī.
	Phospho S129	2	61.81	SNSPPAL	GPEACPVS	LP <mark>S</mark> PPEAST	LK F	Phospho (S1) Phospho (S18)	= 50% ) =92%		2	
		1	49.06	SNSPPAL	GPEACPVS	LPSPPEAST	LK F	Phospho (S3)	= 50%		3	
	Phospho S131	2	61.81	SNSPPAL	GPEACPVS	LP <mark>S</mark> PPEAST	LK F	Phospho (S3) Phospho (S18)	= 50% = 92%		1	
		3 44.			GPEACPVS	LP <mark>S</mark> PPEAST	LK P	hospho (S18)	= 91%		1	
	Phospho S146	2	61.81	SNSPPAL	GPEACPVS	LP <mark>S</mark> PPEAST	LK P	Phospho (S1) Phospho (S18)	= 50% = 92%		2	
	2		61.81	SNSPPALGPEACPVSLPSPPEASTLK Phospho (S3) : Phospho (S18)			= 50% = 92%		1			
	Phospho S165 4 41.63 SPGNLTER				P	hospho (S1) =	= 100%		2			
	Phospho S312	5	38.87	EA	APATGP <mark>S</mark> P(	QHPQK	F	Phospho (S8)	= 89%		1	
С	Exon1 CTCTTGGCTGT	GCACGC	Exon2	AGGCCGCTG	D SHARP HO				EV6		50 120	
Е	35A	ZA	5A	ZA	F						<i>M</i> <sub>r</sub> (K)	
Re SHAF αTu	scue: ≧ ⋚ 6 PI RPIN⊵	S31	0 + EV + + WT + S16	5 5 7 + + + 50	NF-kB-Luc (Relative to WT 0 00 01 0	• WT • KO NT	°°° <sup>8</sup> °°° <sup>8</sup> PI	25 20 15 10 5 0 NT	¢ 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8 8	8 6 4 2 3 0	NT	
G	E6.1 EV EV	WT S165₽	S165L	<i>m<sub>r</sub></i> (( <b>x</b> )					CD2	8		
SHARPIN												
HC GA	DIL-1		<b>5</b> 0 <b>1</b> 7 <b>1</b> 7									
	_	Rescue	•									





Gene Symbol	Fold regulation	P value	Gene Symbol	Fold regulation	P value
SELE	-3.84	0.002010	CXCL2	-2.38	0.024300
CSF3	-3.37	0.003848	IL12B	-2.92	0.178692
II 12B	-2 65	0.011335	CXCL8	-2.83	0.147931
	2.00	0.011000	IL6	-2.82	0.203720
IFNB1	-2.59	0.019553	IL1B	-2.73	0.096160
CXCL1	-2.58	0.006250	SELE	-2.60	0.149616
IL6	-2.34	0.002069	CCND1	-2.34	0.207953
			CXCL9	-2.32	0.093671
PTGS2	-2.26	0.021027	IL4	-2.12	0.181482
CXCL8	-2.22	0.003132	IL1A	-2.09	0.243840
CXCL9	-2.19	0.027996	AGT	-2.05	0.109868
CCL22	-2.13	0.003650			
CXCL2	-2.07	0.029537			
IL2	-2.07	0.027333			

IL4

CCL11

AGT

-2.07

-2.05

-2.04

0.021390

0.018191

0.003451