E3 ubiquitin ligase SYVN1 is a key positive regulator for GSDMD-mediated pyroptosis

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19 **Abstract**

20 Gasdermin D (GSDMD) participates in activation of inflammasomes and pyroptosis.
 21 Meanwhile, ubiquitination strictly regulates inflammatory responses. However, how
 22 ubiquitination regulates Gasdermin D activity is not well understood. In this study, we
 23 show that pyroptosis triggered by Gasdermin D is regulated through ubiquitination.
 24 Specifically, SYVN1, an E3 ubiquitin ligase of gasdermin D, promotes
 25 GSDMD-mediated pyroptosis. SYVN1 deficiency inhibits pyroptosis and subsequent
 26 LDH release and PI uptake. SYVN1 directly interacts with GSDMD, and mediates
 27 K27-linked polyubiquitination of GSDMD on K203 and K204 residues, promoting
 28 GSDMD-induced pyroptotic cell death. Thus, our findings revealed the essential role
 29 of SYVN1 in GSDMD-mediated pyroptosis. Overall, GSDMD ubiquitination is a
 30 potential therapeutic module for inflammatory diseases.

31 **Keywords:** GSDMD; Pyroptosis; Ubiquitination; SYVN1

32

33 **Introduction**

34 Pyroptosis is a form of programmed cell death, characterized by cell swelling, pore
 35 formation in cell membrane and cell lysis, releasing the cytoplasmic contents [1-4]. It
 36 plays an important role in host defense and inflammatory responses [5]. Recent
 37 studies show that pyroptosis is induced by proteolytic cleavage of Gasdermin D
 38 (GSDMD) by inflammatory caspases [2, 6, 7]. Canonical inflammasomes, including
 39 NLRP3 inflammasome, activate caspase-1. Meanwhile, lipopolysaccharide (LPS)
 40 activates non-canonical inflammasomes via the Caspase-11 in marine animals or

41 Caspase-4 and -5 pathways in humans [8-12]. As the final downstream effector of
42 inflammasomes activation, GSDMD is cleaved by inflammatory Caspases at the
43 junction between the N-terminal cytotoxic domain (GSDMD-p30) and C-terminal
44 autoinhibitory domain (GSDMD-p20) [5, 13, 14]. GSDMD-p30 then binds (cellular)
45 phospholipids and oligomerizes to form 10-20 nm pores on the plasma membrane,
46 triggering pyroptotic cell death [15-19].

47 Post-translational modification of inflammasome components via ubiquitin (Ub) is
48 critical for regulation of inflammasomes activation [20, 21]. Ubiquitination of NLRP3,
49 Caspase-1/11, ASC, IL-1 β and other inflammasome components regulates several
50 essential nodes in the regulatory networks [20-27]. E3 ubiquitin ligases such as
51 Pellino 2 mediates K63-linked polyubiquitination and NLRP3 inflammasome
52 activation [28]. In addition, HUWE1 stimulates inflammasomes activation by
53 promoting K27-linked polyubiquitination of NLRP3, AIM2, and NLRC4, which
54 strengthen host defense against bacterial infection [29]. Gasdermin B (GSDMB), a
55 member of gasdermin family, has also been recently found to participates in
56 ubiquitination-mediated pyroptosis that blocks bactericidal functions of NK cells [30].
57 Given that pyroptosis regulation is important in signaling of cell death, GSDMD
58 ubiquitination may broadly be thought to regulate the function of GSDMD and the
59 activities of different inflammasomes [31]. Importantly, the function and mechanisms
60 underlying post-translational modifications of GSDMD such as ubiquitination,
61 deubiquitination and phosphorylation are still unknown.

62 Synoviolin (SYVN1), also known as Hrd1, is one of the RING E3 ligases [32].

63 Given that SYVN1 target numerous substrates such as a pro-apoptotic factor (IRE1)
64 [33], B lymphocyte-induced maturation protein 1 (BLIMP1) [34] and mitochondrial
65 antiviral signaling (MAVS), it performs distinct functions in different cells [35].
66 Furthermore, SYVN1 regulates ER-stress-induced cell death by promoting
67 ubiquitination and degradation of IRE1. Overall, this regulates survival and death of
68 cells [33]. Moreover, SYVN1 promotes T-cell immunity [36] , B-cell immunity [37]
69 and regulates TLR-induced inflammation through K27-linked ubiquitination and
70 inactivation of Usp15 [38]. However, the role of SYVN1 in pyroptosis is not well
71 understood.

72 In this study, we found SYVN1 promotes canonical and non-canonical pyroptosis
73 through GSDMD ubiquitination. Particularly, SYVN1 induces GSDMD-induced
74 pyroptotic cell death by promoting K27-linked polyubiquitination of GSDMD on
75 K203 and K204 residues. Our findings revealed a new mechanism of pyroptotic cell
76 death through post-translational modification of GSDMD.

77

78 **Results**

79 **Ubiquitination of GSDMD**

80 Prior this study, GSDMD ubiquitination and its effect on pyroptosis had not been
81 reported. Herein, HEK293T cells were first transfected with plasmids expressing
82 HA-ubiquitin and Flag-GSDMD. Immunoprecipitation was performed using anti-Flag
83 antibody, whereas western blot was performed using anti-HA or anti-Flag. We found
84 co-expression of HA-ubiquitin and Flag-human GSDMD induced ubiquitination of

85 human GSDMD (Fig. 1A). Further studies confirmed that both mouse and porcine
86 GSDMD proteins can also be ubiquitinated (Fig. 1B, 1C). Further experiments
87 revealed that endogenous human GSDMD can also be modified by ubiquitination (Fig.
88 1D, 1E). Interestingly, LPS/nigericin or TLR1/2 ligand Pam3CSK4 treatment
89 followed by LPS transfection substantially increased ubiquitination of GSDMD in
90 THP-1 cells, which activated canonical and non-canonical inflammasomes (Fig.
91 1D-G). Accordingly, we hypothesized that GSDMD ubiquitination is a critical and
92 efficiently regulated process.

93 **Identification of SYVN1 as a GSDMD-interacting E3 ligase**

94 Since E3 ligase plays an indispensable role in ubiquitination process, we explored the
95 involvement of this enzyme in GSDMD ubiquitination. Analysis of UbiBrowser
96 database revealed that SYVN1 is one of the most important E3 ligases involved in
97 GSDMD ubiquitination (Fig. S1A). HEK293T cells were therefore transfected with
98 Myc-tagged SYVN1 and Flag-GSDMD. We found GSDMD interacted with SYVN1
99 (Fig. 2A). IP $\square$ LC $\square$ MS/MS analysis revealed comparable findings (Fig. 2D, S1B).
100 Further analyses revealed that GSDMD also interacted with SYVN1 in THP-1 cells
101 (Fig. 2B, 2C). Indirect immunofluorescence further revealed that interaction between
102 SYVN1 and human GSDMD occurred in the cytoplasm (Fig. 2E). In general,
103 interaction between SYVN1 and GSDMD is both endogenous and exogenous.

104 **Reconstruction of canonical and non-canonical pyroptosis *in vitro***

105 Given that GSDMD is the key effector of pyroptosis downstream of activated
106 canonical and non-canonical inflammasomes [5, 6], we evaluated the role of GSDMD

ubiquitination on pyroptosis. Since HEK293T cells do not express endogenous GSDMD [39, 40], we reconstructed canonical and non-canonical pyroptosis in HEK293T cells. Expression of both GSDMD and Caspase1/4 significantly induced LDH production (Fig. 3A, 3B). Western blot revealed that Caspase-1 and Caspase-4 cleaved the full-length GSDMD into active N-terminal GSDMD-p30 domain (Fig. 3A, 3B) and obvious propidium iodide (PI) staining was observed by fluorescence microscopy (Fig. 3C, 3D). Same results were observed in cell death and PI staining analyses of GSDMD-p30 triggered pyroptosis (Fig. 3A-D). However, the expression of GSDMD-p30 was not detectable, in agreement with the hypothesis that the protein might have a toxic effect on the host cell [41]. Overall, these results show that the canonical and non-canonical pyroptosis were successfully reconstructed *in vitro*.

SYVN1 promotes GSDMD-mediated pyroptosis

To determine whether SYVN1 regulates GSDMD-mediated pyroptosis, we evaluated LDH release and PI staining (red) based on the reconstruction of canonical and non-canonical pyroptosis. SYVN1 overexpression markedly increased canonical and non-canonical pyroptosis of cells under expressing Caspase-1/4 and GSDMD (Fig. 4A, 4D and S2A). To confirm that SYVN1 performed its function independent of Caspase-1/4, Caspase1/4 was transfected into HEK293T cells 12 h after GSDMD and SYVN1 co-transfection. Pyroptosis was assessed based on LDH release and PI staining (red). LDH release and PI staining were significantly increased compared with the group without SYVN1 after 6 h, 18 h and 24 h of transfection (Fig. 4B, S2B-2C). In addition, SYVN1 interacted with GSDMD but not with Caspase1/4 (Fig.

4H). Accordingly, we speculated that SYVN1 promotes pyroptosis by directly targeting GSDMD. To validate this hypothesis, we evaluated whether direct SYVN1 targeting of GSDMD N-terminal (GSDMD-p30) induces pyroptosis. Of note, transfection of SYVN1 with GSDMD-p30 markedly enhanced LDH release and PI uptake compared with the group without SYVN1 transfection (Fig. 4C, 4D). These results demonstrated that SYVN1 accelerates pyroptosis mainly by targeting the GSDMD-p30 fragment. To further investigate whether E3 ubiquitin ligase activity of SYVN1 is responsible for regulating GSDMD-induced pyroptosis, we assessed the effects of SYVN1 and SYVN1-C329S on pyroptosis in HEK293T cells. Based on previous reports, the cysteine residue at position 329 is critical, and its mutation to serine (C329S) abolishes SYVN1 E3 activity [38, 42]. We found SYVN1-C329S which lacks E3 ligase activity could not increase LDH release (Fig. 4E-G). To determine whether GSDMD ubiquitination activates pyroptosis, HEK293T cells were co-transfected with GSDMD-p30 and wild-type Ub. It was found that overexpression of Ub significantly promoted GSDMD-p30-mediated pyroptosis (Fig. S2D, S2E), indicating that ubiquitination of GSDMD facilitates pyroptosis. Importantly, the addition of Ub further promoted SYVN1 enhanced GSDMD-p30-mediated pyroptosis (Fig. 4I). Collectively, these findings demonstrated that SYVN1 promotes pyroptosis through ubiquitination of GSDMD.

Inhibition of SYVN1 impairs GSDMD-mediated pyroptosis

To further confirm the role of SYVN1 on pyroptosis, we designed three siRNAs targeting SYVN1. The efficiency of SYVN1 knock-down was shown in Fig. 5A, and

we ultimately selected siSYVN1 #3 for further studies because of its better knockdown efficacy (Fig. 5A). LDH assay and PI uptake revealed that SYVN1 knockdown inhibited the effect of GSDMD-p30 on pyroptosis in HEK293T cells (Fig. 5B, 5C, S3). To further investigate the role of SYVN1 in pyroptosis, we generated SYVN1 knockout HEK293T cells by CRISPR/Cas9-mediated genome editing (according to reference [43]). The efficiency of SYVN1 knock-out was shown in Fig. 5D, and we finally selected sgSYVN1 #2 for further research because of its better knockout effect. Consistent with the results of knockdown of SYVN1, knockout of SYVN1 in HEK293T cells significantly suppressed canonical, non-canonical pyroptosis and GSDMD-p30 activation (Fig. 5E). Collectively, these findings further demonstrated that SYVN1 promotes GSDMD-mediated pyroptosis.

SYVN1 ubiquitinates GSDMD with K27-linked polyubiquitin chains

Considering that SYVN1 regulate pyroptosis in an E3 ligase activity-dependent manner, we speculated SYVN1 mediates polyubiquitination of GSDMD. As such, we co-expressed Myc-SYVN1-WT or the E3 ligase-dead mutant SYVN1 C329S with HA-ubiquitin (HA-Ub) and Flag-GSDMD in HEK293T cells. We found compared to C329S mutant, overexpression of SYVN1-WT substantially increased GSDMD ubiquitination (Fig. 6A), suggesting that E3 ubiquitin ligase activity is necessary for SYVN1 ubiquitination of GSDMD. Since K48 and K63 are the most studied canonical ubiquitination, we further investigated the role of these two polyubiquitination chains in ubiquitination of GSDMD. HEK293T cells were transfected with Flag-GSDMD and HA-K48-ubiquitin, HA-K63-ubiquitin,

173 HA-K48R-ubiquitin or HA-K63R-ubiquitin. It was found SYVN1 had no effect on
 174 K48 and K63-mediated polyubiquitination of GSDMD (Fig 6B). Also, SYVN1
 175 promoted K48R and K63R-mediated polyubiquitination, suggesting that SYVN1
 176 induced addition of other ubiquitination chains on GSDMD. Given that seven lysine
 177 residues (K6, K11, K27, K29, K33, K48 and K63) have been reported to form
 178 polyubiquitin chains [44-48], we sequentially co-transfected these HA-tagged
 179 ubiquitin mutants (with only one of the seven lysine residues retained as lysine, and
 180 the other six replaced with arginine) with Flag-tagged GSDMD with or without
 181 SYVN1 into HEK293T cells. Co-immunoprecipitation assay revealed that K27
 182 ubiquitin mutant markedly increased polyubiquitination of GSDMD (Fig. 6C).
 183 However, overexpression of SYVN1 had no effect on GSDMD polyubiquitination in
 184 K27R mutant transfected cells (Fig. 6D). These findings suggest that SYVN1
 185 promoted GSDMD-mediated pyroptosis may through K27-linked polyubiquitination
 186 of GSDMD. Indeed, overexpression of Ub-K27 promoted pyroptosis induced by
 187 Caspase-1/4 mediated GSDMD cleavage and GSDMD-p30 activation (Fig. 6E-F).
 188 Thus, SYVN1 mediates K27-linked polyubiquitination of GSDMD.

189 **K203 and K204 residues of GSDMD are polyubiquitinated by SYVN1**

190 To determine sites of GSDMD polyubiquitination by SYVN1, ubiquitination in
 191 HEK293T cells was induced through expression of Flag-GSDMD, SYVN1-Myc, and
 192 HA-ubiquitin. Controls expressed SYVN1-C329S-Myc, Flag-GSDMD and
 193 HA-ubiquitin. After immunoprecipitating GSDMD with anti-Flag purification beads,
 194 the protein was separated using SDS-PAGE and visualized after Coomassie blue

195 staining (Fig. 7A). To analyze ubiquitinated GSDMD sites by mass spectrometry
 196 (MS), the gel regions (Fig. 7A) which might contain ubiquitin-modified proteins were
 197 excised, digested with trypsin, and analyzed by liquid chromatography-MS. A
 198 database search of the MS/MS spectra revealed that GSDMD is the predominant
 199 protein identified in the samples. Representative spectra demonstrated that the K51
 200 and K299 residues of GSDMD in gel were modified with ubiquitin (Fig. S4A). We
 201 also used the UbPred program (<http://www.ubpred.org>) [49-51], a predictor of
 202 potential ubiquitin binding sites in proteins to predict the potential ubiquitination sites
 203 of GSDMD. This analysis revealed other five putative target lysine residues: K103,
 204 K203, K204, K235 and K248 (Fig. S4B). We then generated Flag-GSDMD K51R,
 205 K103R, K203R, K204R, K235R, K248R and K299R mutants in which each of these
 206 lysine residues was replaced with arginine (R). To determine if these potential
 207 ubiquitination sites altered pyroptotic function, HEK293T cells were co-transfected
 208 with wild-type GSDMD or Flag-GSDMD mutants and Caspase1/4. LDH release and
 209 pore formation assay revealed that pyroptosis was significantly low in
 210 GSDMD-K103R, GSDMD-K203R, GSDMD-K204R, GSDMD-K235R and
 211 GSDMD-K248R mutants, but not in GSDMD-K51R and K299R mutants (Figs. 7B,
 212 7C, S5A). GSDMD-D275A, which is the only cleavage site of Caspase1/4, was used
 213 as the positive control [5, 13]. As expected, Caspase-1/4 did not cleave
 214 GSDMD-D275A, and the mutant (GSDMD-D275A) had no effect on pyroptosis (Figs.
 215 7B, 7C). Moreover, mutations in GSDMD-p30 ubiquitination sites substantially
 216 decreased GSDMD-p30-mediated pyroptosis (Fig. 7D , S5B). GSDMD-p30-C191A

217 was used as a positive control, given that mutation (C191A) in this amino acid
218 decreases oligomerization and inhibits pyroptosis [40, 52, 53]. Our results suggest that
219 amino acids GSDMD-K103, GSDMD-K203, GSDMD-K204, GSDMD-K235 and
220 GSDMD-K248 are critical for pyroptosis.

221 To further explore SYVN1-mediated ubiquitination sites on GSDMD, HEK293T
222 cells were transfected with Myc-tagged SYVN1, Flag-tagged GSDMD or GSDMD
223 K/R mutants and HA-tagged ubiquitin. Compared with the WT GSDMD (Fig. 7E,
224 lane 1), SYVN1 had no effect on ubiquitination of GSDMD-K51R or K299R mutant
225 (Fig. 7E, lane 7, 8). In contrast, even though LDH decreased in GSDMD K103R,
226 K235R and K248R mutants (Fig. 7B, 7C), SYVN1-mediated ubiquitination of
227 GSDMD occurred in them (Fig. 7E, lane 2, 5, 6). Specifically, Co-IP assay revealed
228 that SYVN1-mediated GSDMD ubiquitination was partially blocked in K203R and
229 K204R mutants (Fig. 7E, lane 3, 4). K203R/K204R mutants were then generated to
230 further confirm K203R and K204R lysine residues are the major ubiquitination sites
231 in GSDMD. We found arginine mutations in these two residues (2KR) did not
232 completely prevent GSDMD ubiquitination (Fig. 7F), suggesting that SYVN1 also
233 modifies other lysine residues. Overall, the above findings show that SYVN1 mainly
234 ubiquitinates GSDMD at K203 and K204 residues, but there are also other lysine
235 residues in GSDMD that are ubiquitinated by SYVN1.

236

237

238

239 **Discussion**

240 Pyroptosis, mediated by the inflammasomes, Caspase-1 and Caspase-4/5/11,
241 participates in the clearance of intracellular pathogens [6, 54]. Recent studies have
242 identified Gasdermin D (GSDMD) as the primary executioner of pyroptosis [5, 6].
243 However, ubiquitination-mediated regulation of GSDMD activity is poorly
244 understood. In this study, we demonstrated that ubiquitination of GSDMD plays a
245 critical role in pyroptosis. Moreover, SYVN1 is an important E3 ubiquitin ligase that
246 positively regulates canonical and non-canonical pyroptosis through K27-linked
247 polyubiquitination of K203 and K204 lysine residues on GSDMD (Fig. 8).

248 Polyubiquitination is a post-translational modification process that plays critical
249 roles in programmed cell death or NLRP3-mediated inflammasome activation through
250 covalent linkage of ubiquitin to lysine residues in target proteins [20, 22, 55, 56].
251 Recent studies have found that GSDMD is essential in pyroptosis. Thus, it is
252 reasonable to hypothesize that polyubiquitination of common components of
253 canonical and non-canonical pyroptosis effectively regulates pyroptosis in response to
254 infection and danger signals. Herein, we demonstrated ubiquitination of GSDMD
255 facilitates GSDMD-mediated pyroptosis.

256 SYVN1 is over-expressed in synoviocytes of patients with rheumatoid arthritis,
257 exacerbating the pathogenesis of the disease [57]. Moreover, SYVN1 regulates the
258 functioning of immune cells, including T cells [36], B cells [37], and macrophages
259 [38]. However, how SYVN1 regulates pyroptosis had not been previously described.
260 We found overexpression of SYVN1 increases LDH release and pyroptosis (Fig. 4),

261 and as such, loss of SYVN1 function impairs GSDMD-mediated pyroptosis (Fig. 5).

262 In general, SYVN1 promotes GSDMD-mediated pyroptosis.

263 There were multiple K residues in GSDMD that can be ubiquitinated based on the
 264 prediction by Ubipred online software. These residues could be ubiquitinated by
 265 SYVN1 and other E3 ubiquitin ligase enzymes, and additional studies to identify
 266 these ubiquitin ligases are needed. Herein, we found SYVN1 promotes pyroptosis
 267 mainly by interacting and ubiquitinating K203 and K204 residues in GSDMD. Mass
 268 spectrometric analysis further revealed two high-confidence ubiquitin modified
 269 residues in the N- (K51) and C-terminal (K299) domains of GSDMD (Fig. S4A).
 270 However, mutation in these residues (K51R/K299R) had no significant effect on
 271 GSDMD ubiquitination, suggesting that SYVN1 also modifies other lysine residues.
 272 Furthermore, a recent study revealed that pyroptotic cell death is independent of
 273 GSDMD-K204T modification [58]. In contrast, we found ubiquitination of
 274 GSDMD-K204R mutant inhibited GSDMD-induced pyroptosis. The discrepancy may
 275 have resulted from the presence of threonine residue, which could also be
 276 ubiquitinated [59]. Further studies are needed to explore how mutations in GSDMD
 277 ubiquitination sites influence pyroptosis *in vivo*.

278 Taken together, findings of this study elucidated the unique role of GSDMD
 279 polyubiquitination in pyroptosis, in particular, mediated by SYVN1. First, we found
 280 GSDMD ubiquitination occurs in several species including human, mouse, and swine.
 281 Second, we found SYVN1, an E3 ubiquitin ligase, is the primary regulator of
 282 GSDMD activity, and performs its function by promoting ubiquitination of GSDMD.

Specifically, SYVN1 directly interacts with GSDMD, and mediates K27-linked polyubiquitination of GSDMD on K203 and K204 residues, promoting GSDMD-induced pyroptotic cell death. Summed together, we report a new mechanism of the growing list ubiquitin-mediated regulation, a noble mechanism of PRR signaling pathways.

Materials and Methods

Reagents

Anti-Flag M2 (F1804), anti-Flag M2 Magnetic Beads, anti-Myc (M5546) and anti-LPS (O11:B4) were purchased from Sigma-Aldrich. Anti-GSDMD (sc-393581) and anti-GAPDH (sc-47724) were from Santa Cruz Biotechnology. Anti-Ub (sc-8017), anti-HA (3724), anti- β -actin (3700) and the secondary antibodies used for western blot and immunofluorescence assay were purchased from Cell Signaling Technology. Anti-SYVN1 (ab170901) was purchased from Abcam, whereas Nigericin, Pam3CSK4 (tlrl-pms) were purchased from InvivoGen. ELISA kits for analysis of human IL-1 β were purchased from MultiSciences. CytoTox 96 LDH-release assay kit (G1780) was purchased from Promega.

Cell culture and stimulation

Human HEK293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) in a humidified incubator at 37°C with 5 % CO₂, supplemented with 10 % FBS (Corille, C1015-05), 100 μ g/ml streptomycin and 100 U/ml penicillin (Hyclone, 347 SV30010). Human monocyte cell line THP-1 was cultured in PRIM 1640

medium supplemented with 10 % FBS, 100 µg/ml streptomycin and 100 U/ml penicillin. To activate canonical inflammasomes, 5×10^5 cells were plated overnight in 24-well plates, and primed for 4 h with 500 ng/ml LPS. The cells were then stimulated for 1 h using 10 µM Nigericin. For non-canonical inflammasome activation, cells were primed for 4 h with 500 ng/ml Pam3CSK4, after which the medium was replaced and cells were transfected with 2 µg/ml LPS using Lipofectamine 2000 for 6 h.

Plasmid construction and transfection

cDNAs for transfection in plasmids were obtained by reverse transcription of total RNA from HEK293T and THP-1 cells. The cDNAs were amplified using specific primers before sub-cloning the PCR products into p3Flag-CMV-7.1, pCMV-Myc and pCDNA-3.1 vectors. HA-tagged ubiquitin was provided by Dr. Yimin Yang (Zhejiang University). The primers used in this study are shown in Supplementary Table 1.

Induction of pyroptosis in HEK293T cells

HEK293T cells grown in 24-well plates to 80% confluence were transfected with 600 ng of p3XFlag-GSDMD and 600 ng of pCMV-Caspase1/4 using VigoFect. The supernatants were collected 24 h after transfection and analyzed for IL-1β level using ELISA.

ELISA

Supernatants from transfected cells were assayed for human IL-1β in accordance with the manufacturer's instructions. Each experiment was performed independently at least three times. Sample preparation for ELISA was according to the method [60].

327 **Cytotoxicity assay and propidium iodide staining**

328 Culture medium of HEK293T cells co-transfected for 24 h with p3Flag-CMV-7.1,
329 pCMV-Myc, and pCDNA-3.1 was collected and assayed for LDH release using the
330 Cytotoxicity Detection Kit (Promega) according to the manufacturer's manual. The
331 HEK293T cells were cultured for 15 min with 2.5 µg/ml Propidium iodide (BD
332 Bioscience, 556463) and observed under a fluorescence microscopy. The experiments
333 were performed independently in triplicate.

334 **Western blot analysis**

335 Cells were lysed using lysis buffer (Beyotime, P0013) supplemented with
336 Phenylmethanesulfonyl fluoride (PMSF) (Beyotime, ST506). Total proteins in the
337 cells were extracted and thereafter separated using 12 % SDS-PAGE. The proteins
338 were then transferred onto PVDF membrane, hybridized with primary antibodies and
339 probed with HRP-conjugated secondary antibodies. Proteins were detected using the
340 image system (Clinx Science Instruments, China).

341 **Co-immunoprecipitation assay**

342 Cells were lysed for 30 min in ice cold lysis buffer and centrifuged at $10,000 \times g$ at
343 4°C. The supernatants were then incubated with anti-Flag binding beads (Sigma,
344 M8823) at 4 °C. The beads were then washed for 3-5 times with cold TBS. Immune
345 complexes were denatured for 10 min at 100 °C in 1 × SDS-PAGE loading buffer
346 before western blot analysis.

347 **Confocal immunofluorescence assay**

348 HEK293T cells were seeded in 24-well at the rate of 1×10^5 per well and transfected

for 24 h with p3Flag-CMV-7.1, pCMV-Myc, and pCDNA-3.1 plasmids. The cells were then fixed for 30 minutes at room temperature with 4% paraformaldehyde (Beyotime, P0098) before incubation with primary antibodies in DAPI (Beyotime, C1002). The cells were then observed using a laser scanning microscope (Olympus, IX81-FV1000).

SYVN1 knockdown using small interfering RNA

Short interfering RNAs (genepharma) specific for human SYVN1 were transfected into THP-1 and HEK293T cells using the Lipofectamine lipo8000 reagent (Beyotime, C0533FT) according to the manufacturer's instructions. The sequences of the siRNAs used in this study are shown in Supplementary Table 2.

Construction of SYVN1 knockout HEK293T using CRISPR/Cas9-technique.

SYVN1 knockout HEK293T cells were generated using CRISPR/Cas9 technique. Vectors expressing gRNA targeting human SYVN1 were transfected into HEK293T cells using manufacturer's protocol. In general, based on flow-cytometric analysis of GFP levels, SYVN1 knockout was achieved in 30-50% of HEK293T cells. Single cell sorting of transfected cells was performed using flow cytometry (MOFLO XDP). gRNA sequences are shown in Supplementary Table 3.

LC-MS/MS analysis

Flag-tagged GSDMD immunoprecipitates prepared from whole-cell lysates or gel-filtrated fractions were resolved on SDS-PAGE gels, and protein bands were excised. The samples were digested with trypsin, and then subject to LC-MS/MS Analysis. Swissprot_Human mass spectra were used as the standard reference.

371 Trypsin/P was used for cleavage. MS data was captured and analyzed using
372 Micrometer Biotech and Maxquant, respectively.

373 **Statistical analysis**

374 The values are presented as mean $\pm$ SD. Data was analyzed using GraphPad Prism 8.0.
375 Difference between experimental groups was assessed using Student's *t*-test or
376 one-way ANOVA. All experiments were performed independently at least three times.
377 Statistical significance was set at $P < 0.05$, 0.01 or 0.001. **P* represents $P < 0.05$,
378 ***P* represents $P < 0.01$ and ****P* represents $P < 0.001$.

379

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391 **Conflict of interest**

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

393 **Figure legends**

394 **Fig. 1 GSDMD can be modified by ubiquitination.**

395 **A**, Lysates from HEK293T cells co-transfected with HA-Ub along with Flag-human
 396 GSDMD (hGSDMD) or not, were subjected to immunoprecipitation with Flag
 397 antibody followed by western blot analysis using anti-HA antibody. **B**,
 398 Immunoprecipitation and western blot analysis of HEK293T cells co-transfected with
 399 HA-Ub and Flag-porcine GSDMD (pGSDMD) using anti-Flag and anti-HA
 400 antibodies, respectively. **C**, Immunoprecipitation and western blot analysis of
 401 HEK293T cells co-transfected with HA-Ub and Flag-mouse GSDMD (mGSDMD)
 402 using anti-Flag and anti-HA antibodies, respectively. **D** , **F** , THP-1 cells were primed
 403 with LPS (500 ng/ml) for 4 h, and then treated with or without Nigericin (10 μ M).
 404 Cell lysates were immunoprecipitated with anti-GSDMD antibody, followed by
 405 western blot analysis with anti-GSDMD and anti-Ub antibodies, respectively.
 406 Secretion of IL-1 β was analyzed using ELISA. LDH release was analyzed using LDH
 407 release assay. **E** , **G** , THP-1 cells were incubated with Pam3CK4 (500 ng/ml) for 4 h,
 408 and then transfected with or without LPS (2 μ g/ml) for 6 h. Cell lysates were
 409 immunoprecipitated with anti-GSDMD antibody, followed by western blot analysis
 410 with anti-GSDMD and anti-Ub antibodies. IL-1 β secretion in THP-1 cells was
 411 analyzed using ELISA, whereas LDH release was analyzed using LDH release assay.

412 **Fig. 2 SYVN1 interacts with endogenous and exogenous GSDMD.**

413 **A**, HEK293T cells co-transfected with pcDNA3.1-SYVN1-Myc and
 414 p3xFlag-GSDMD were lysed and immunoprecipitated with anti-Flag antibody. Both

the immunoprecipitates (IP) and whole cell lysates (Input) were subjected to gel electrophoresis and analyzed by western blot with anti-Myc and anti-Flag antibodies, respectively. **B-C**, Immunoprecipitation analysis of THP-1 cells using anti-SYVN1 or anti-GSDMD antibodies. Western blot of both IP and total proteins in THP-1 cells was performed using anti-SYVN1 and anti-GSDMD antibodies, respectively. **D**, HEK293T cells co-transfected with pcDNA3.1-SYVN1-Myc and p3xFlag-GSDMD were lysed and immunoprecipitated with anti-Flag antibody. The potential GSDMD-binding proteins in HEK293T cells were evaluated using Co-IP and MS analysis. The MS/MS spectrum of 71-AAEMEHLER-80 is shown. Observed b- and y-ion series are indicated. **E**, Immunostaining analysis of HEK293T cells after transfection with pcDNA3.1-SYVN1-Myc and p3xFlag-GSDMD using anti-Flag and anti-Myc antibodies. Subcellular localizations of Flag-GSDMD (green), Myc-SYVN1 (red), and DAPI (blue), a nucleus marker, were observed using a confocal microscopy. Scale: 1 bar represents 10 μ m.

Fig. 3 Reconstruction of canonical and non-canonical pyroptosis *in vitro*.

A, HEK293T cells were co-transfected with pCMV-Myc-Caspase-1 (600 ng) and p3XFlag-hGSDMD-FL (600 ng). pcDNA3.1-hGSDMD-p30-Myc (600 ng) was used as a positive control. Supernatants were analyzed using LDH assay. Cell lysates were normalized for proteins contents and analyzed using western blot using antibodies specific for Flag, Myc and GAPDH. **B**, HEK293T cells were co-transfected with pCMV-Myc-Caspase-4 and p3XFlag-hGSDMD-FL. The pcDNA3.1-hGSDMD-p30-Myc was used as positive controls. Anti-Flag, Myc and

437 GAPDH antibodies were used for western blot analyses. **C-D**, Pyroptosis of
438 HEK293T cells after GSDMD cleavage as observed under bright-field and
439 epifluorescent microscopy. Scale: 1 bar represents 100 μ m.

440 **Fig. 4 Overexpression of SYVN1 promotes GSDMD-triggered pyroptosis.**

441 **A, D**, HEK293T cells were transfected with pCMV-Myc-Caspase-1/4 (100 ng),
442 p3XFlag-hGSDMD-FL (200 ng) and p3XFlag-SYVN1 (600 ng) or control vector.
443 The supernatants were collected and analyzed by LDH release assay and cell staining
444 with PI after 24 h transfection. PI analysis was performed using 2.5 μ g/ml. Scale: 1
445 bar represents 20 μ m. **B**, HEK293T cells were transfected with Myc-SYVN1 (600 ng)
446 and p3XFlag-hGSDMD-FL (200 ng) for 12 h, and then transfected with Caspase-1/4
447 (100 ng) for 6 h. **C-D**, HEK293T cells were transfected with Myc-SYVN1 (600 ng)
448 and hGSDMD-p30-Myc (100 ng). Supernatants were collected and analyzed by LDH
449 release assay and cell staining with PI at 24 h after transfection. **E-F**, LDH release
450 assay of HEK293T cells after co-transfection with Caspase-1/4 (100 ng),
451 hGSDMD-FL (200 ng) and SYVN1-WT (600 ng) or SYVN1-C329S (600 ng). **G**,
452 LDH release assay of HEK293T cells co-transfected with hGSDMD-p30 (100 ng) and
453 SYVN1-WT (600 ng) or SYVN1-C329S (600 ng). The supernatants were collected
454 and analyzed by LDH release assay at 24 h after transfection. **H**, Immunoprecipitation
455 (IP) and immunoblot (IB) analysis of HEK293T cells were co-transfected with
456 pcDNA3.1-SYVN1-Myc and p3XFlag-hGSDMD-FL or p3XFlag-Caspase-1/4. IP
457 was performed using anti-Flag antibody, whereas IB was performed using anti-Myc
458 antibody. **I**, LDH release of HEK293T cells co-transfected with hGSDMD-p30,

459 SYVN1 and Ub. The analysis was performed at 24 h after transfection.

460 **Fig. 5 Effect of inhibiting SYVN1 on GSDMD-mediated pyroptosis.**

461 **A**, Western blot analysis for efficiency of SYVN1 expression inhibition in HEK293T
 462 cells using siSYVN1. The analysis was performed using anti-SYVN1 antibody. **B-C**,
 463 HEK293T cells were transfected siSYVN1-control (50 nM) or siSYVN1 (50 nM), 48
 464 h after transfection, cells were transfected with hGSDMD-p30. The supernatants were
 465 collected and analyzed by LDH release assay. After 24 h transfection, the cells were
 466 staining with PI (2.5 µg/ml) and analyzed by Fluorescence microscopy. **D**, The
 467 efficiency of sg-SYVN1 was analyzed by western blot with anti-SYVN1 antibody in
 468 HEK293T cells obtained from WT and SYVN1-KO HEK293T cells. **E**, HEK293T
 469 WT and SYVN1-KO cells were transfected with Caspase-1/4 and hGSDMD-FL or
 470 hGSDMD-p30 alone. The supernatants were collected and analyzed by LDH release
 471 assay.

472 **Fig. 6 SYVN1 ubiquitinates GSDMD with K27-linked polyubiquitin chains.**

473 **A**, Immunoprecipitation analysis in HEK293T cells co-expressing Flag-GSDMD and
 474 HA-Ub together with Myc-SYVN1 or Myc-SYVN1-C329S. Anti-Flag
 475 immunoprecipitates were analyzed using western blot with indicated antibodies. The
 476 expression levels of the transfected proteins were analyzed using western blot with
 477 indicated antibodies. **B**, Immunoprecipitation analysis of HEK293T cells
 478 co-expressing Flag-GSDMD and Myc-SYVN1 together with HA-Ub (K48, K63,
 479 K48R or K63R) as indicated. Western blot analysis was performed using anti-Myc or
 480 anti-Flag antibodies. Expression of the transfected proteins was analyzed using

western blot, using corresponding antibodies. **C**, Immunoprecipitation analysis of HEK293T cells expressing Flag-GSDMD and Myc-SYVN1 together with HA-Ub (K6, K11, K27, K29, K33, K48, or K63 only) as indicated. Anti-Flag immunoprecipitates were analyzed using western blot with anti-Myc or anti-Flag antibodies. Expression of the transfected proteins was analyzed using western blot, using corresponding antibodies. **D**, Immunoprecipitation analysis of HEK293T cells expressing Flag-GSDMD and Myc-SYVN1 together with HA-Ub (K27 or K27R) as indicated. Anti-Flag immunoprecipitates were analyzed using western blot with indicated antibodies. Expression of the transfected proteins was analyzed using western blot, using corresponding antibodies. **E**, LDH release assay of HEK293T cells transfected with Caspase-1/4, GSDMD and Ub-K27 or control vector. **F**, LDH release assay of HEK293T cells transfected with hGSDMD-p30 and Ub-K27 or control vector.

Fig. 7 SYVN1 polyubiquitinates K203 and K204 residues of GSDMD .

A, Coomassie blue-stained bands of polyubiquitinated GSDMD. HEK293T cells were co-transfected with Flag-GSDMD, Myc-SYVN1 or Myc-SYVN1-C329S and HA-ubiquitin (shown above the lanes). The square areas indicate regions from which proteins were removed for MS. **B-C**, LDH assay of HEK293T cells transfected with pCMV-Myc-Caspase-1/4 (300 ng), p3XFlag-hGSDMD-FL (600 ng) or GSDMD mutant (600 ng). The analysis was performed at 24 h after transfection. **D**, LDH release assay of HEK293T cells transfected with GSDMD-p30 (300 ng) or GSDMD-p30 mutants (300 ng). The analysis was performed at 24 h after transfection.

503 **E**, Immunoprecipitation analysis of HEK293T cells expressing Flag-GSDMD or
 504 Flag-GSDMD mutants together with HA-Ub and Myc-SYVN1. Expression of
 505 proteins of interest was analyzed using western blot, using corresponding antibodies.
 506 Densitometry of the blots was measured using Image J. **F**, Immunoprecipitation
 507 analysis of HEK293T cells expressing Flag-GSDMD, K203R, K204R or 2KR
 508 mutants along with HA-Ub-K27 and Myc-SYVN1. Western blot was performed using
 509 corresponding antibodies.

510 **Fig. 8 Diagram of SYVN1 regulates GSDMD-mediated pyroptosis.**

511 **Supplementary Figure 1 A**, Prediction of GSDMD ubiquitination by E3 ubiquitin
 512 ligase using the Ubibrowser software. **B**, MS/MS analysis for representative
 513 sequences of Human SYVN1 alleles.

514 **Supplementary Figure 2 A**, Bright-field and epifluorescent microscopy of HEK293T
 515 cells after PI staining. Scale: 1 bar represents 50 μ m. **B**, HEK293T cells were
 516 transfected with Myc-SYVN1 and p3XFlag-hGSDMD-FL and thereafter for 18 h or
 517 24 h with Caspase-1/4. **C**, HEK293T cell were transfected with plasmids as shown. PI
 518 analysis was performed after 24 h transfection. Scale: 1 bar represents 50 μ m. **D-E**,
 519 HEK293T cells transfected with varied dose of hGSDMD-p30 and Ub. The
 520 supernatants were collected and analyzed after 24 h transfection for LDH release
 521 assay (D). HEK293T cells were observed under bright-field and epifluorescent
 522 microscopy after PI staining (E). Scale: 1 bar represents 100 μ m.

523 **Supplementary Figure 3**, HEK293T cells under a fluorescence microscopy after PI
 524 staining. The cells were first transfected with siSYVN1 (50 nM) or corresponding

control and thereafter with hGSDMD-p30 after 48 h. Scale: 1 bar represents 50 μ m.

Supplementary Figure 4 Putative sites on hGSDMD. **A**, Representative MS/MS spectra of ubiquitinated hGSDMD peptides. Protein samples were recovered from the gel, enzymatically digested and analyzed using Mass Spectrometry (MS). **B**, Ubiquitination patterns of GSDMD. Red areas represent ubiquitination sites predicted by Ubibrowser software, whereas the green sites represent ubiquitination sites identified by MS.

Supplementary Figure 5 A-B, PI staining analysis of HEK293T cells after 24 h transfection with indicated plasmids. Scale: 1 bar represents 50 μ m.

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