

Host E3 ubiquitin ligase ITCH mediates *Toxoplasma gondii* effector GRA35-triggered NLRP1 inflammasome activation and cell-autonomous immunity.

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Author Contributions

Y.W. and J.P.J.S. designed experiments and wrote the manuscript with input from all authors. Y.W. performed and interpreted most of the experimental work. L.O.S., T.C.P.S., and S.K. helped with the CRISPR screen. L.R.H. produced all the recombinant proteins and performed *in vitro* ubiquitination assays under the supervision of W.H. B.H.P. contributed reagents and equipment and provided input on the design of the experiments.

32 **ABSTRACT:**

33 *Toxoplasma gondii* is an intracellular parasite that can activate the NLRP1 inflammasome leading to
34 macrophage pyroptosis in Lewis rats, but the underlying mechanism is not well understood. In this study,
35 we performed a genome-wide CRISPR screen and identified the dense granule proteins GRA35, GRA42,
36 and GRA43 as the *Toxoplasma* effectors mediating cell death in Lewis rat macrophages. GRA35 localizes
37 on the parasitophorous vacuole membrane, where it interacts with the host E3 ubiquitin ligase ITCH.
38 Inhibition of proteasome activity or ITCH knockout prevented pyroptosis in *Toxoplasma*-infected Lewis rat
39 macrophages, consistent with the "NLRP1 functional degradation model". However, there was no evidence
40 that ITCH directly ubiquitinates or interacts with rat NLRP1. We also found that GRA35-ITCH interaction
41 affected *Toxoplasma* fitness in IFN γ -activated human fibroblasts, likely due to ITCH's role in recruiting
42 ubiquitin and the parasite-restriction factor RNF213 to the parasitophorous vacuole membrane. These
43 findings identify a new role of host E3 ubiquitin ligase ITCH in mediating effector-triggered immunity, a
44 critical concept that involves recognizing intracellular pathogens and initiating host innate immune
45 responses.

46
47 **IMPORTANCE:**

48 Effector-triggered immunity represents an innate immune defense mechanism that plays a crucial role in
49 sensing and controlling intracellular pathogen infection. The NLRP1 inflammasome in the Lewis rats can
50 detect *Toxoplasma* infection, which triggers proptosis in infected macrophages and eliminates the
51 parasite's replication niche. The work reported here revealed that host E3 ubiquitin ligase ITCH is able to
52 recognize and interact with *Toxoplasma* effector protein GRA35 localized on the parasite-host interface,
53 leading to NLRP1 inflammasome activation in Lewis rat macrophages. Furthermore, ITCH-GRA35
54 interaction contributes to the restriction of *Toxoplasma* in human fibroblasts stimulated by IFN γ . Thus, this
55 research provides valuable insights into understanding pathogen recognition and restriction mediated by
56 host E3 ubiquitin ligase.

57
58 **KEYWORDS:**

59 E3 ubiquitin ligase, Effector-triggered immunity, NLRP1 inflammasome, IFN γ , *Toxoplasma gondii*

61 INTRODUCTION:

62 The innate immunity system is the body's first line of defense against infections, with cells of the innate
63 immune system constantly recognizing infections through pattern recognition receptors (PRRs) and
64 coordinating cellular and molecular mechanisms to mount effective antimicrobial responses. In response to
65 particular pathogens, mammalian cells possess a sophisticated recognition mechanism named effector-
66 triggered immunity (1). Effector-triggered immunity occurs when certain intracellular PRRs, known as Nod-
67 like Receptors (NLRs), sense specific effectors secreted during the infection of pathogenic microbes or the
68 alterations they induce after breaching host cell barriers. Upon detection, these NLRs can assemble into
69 multiprotein complexes referred to as inflammasomes, which can be found in various immune cells and
70 play critical roles in initiating host defense against infections (2). Upon recognition of pathogen effectors by
71 inflammasome sensors, inflammatory caspases (Caspase-1, -4, or -11) are recruited and activated, leading
72 to the release of IL1 β and IL18 from infected cells (3), and inducing a form of programmed cell death known
73 as pyroptosis via the cleavage and activation of a pore-forming protein called Gasdermin D (4, 5). Thus, the
74 activation of inflammasomes primarily contributes to the rapid elimination of invading pathogens and is
75 central for the mammalian innate immunity system in triggering inflammation and engaging the adaptive
76 immune system for a more precise response.

77 The nucleotide-binding domain, leucine-rich repeat-containing proteins family, pyrin domain
78 containing 1 (NLRP1) is the first NLR discovered to form an inflammasome (3). The NLRP1 inflammasome
79 is activated by various pathogen effectors through a mechanism of "functional degradation" (6–8). The
80 NLRP1 protein undergoes autoproteolytic processing within its function-to-find (FIIND) domain (9, 10),
81 resulting in two polypeptides (N-terminal and C-terminal) that remain associated in an autoinhibited state.
82 The autoinhibitory N-terminal NLRP1 polypeptide can be ubiquitinated by pathogen E3 ubiquitin ligases,
83 such as *Shigella flexneri* IpaH7.8 (6), or processed by other pathogen proteases (e.g., *Bacillus anthracis*
84 lethal factor and enteroviral 3C protease), leading to its ubiquitination by host N-end rule E3 ubiquitin
85 ligases (6–8, 11, 12). This allows the active C-terminal NLRP1 polypeptide to dissociate upon proteasomal
86 degradation of the N-terminal polypeptide and subsequently recruit Caspase-1 for inflammasome
87 activation^{12–14}. This activation process indicates that NLRP1 generally acts as a guard to sense the specific
88 activity induced by pathogen effectors.

89 *Toxoplasma gondii* is an obligated intracellular pathogen and a highly successful parasite that can
90 infect any nucleated cell and causes lifelong chronic infections in almost all warm-blooded animals. In
91 humans, *Toxoplasma* can cause congenital infections and opportunistic infections in immunocompromised
92 individuals (13). Although *Toxoplasma* possesses an extraordinary host range, the Lewis (LEW) rat is the
93 only known warm-blooded animal with sterilizing immunity against the parasite (14). Polymorphisms in the
94 rat *Nlrp1* gene determine rat strain differences in susceptibility to *Toxoplasma*. The parasite specifically
95 activates the LEW rat NLRP1 inflammasome, resulting in macrophage pyroptosis and subsequent
96 clearance of the infection (15–17). Moreover, NLRP1 plays a role in human monocyte control of
97 *Toxoplasma*, and polymorphisms in *NLRP1* also influence the severity of congenital toxoplasmosis (18).
98 However, the exact mechanism by which *Toxoplasma* activates the NLRP1 inflammasome remains
99 unknown.

100 The key to the parasite's successful survival and proliferation in diverse host cell microenvironment
101 is that *Toxoplasma* resides within a non-fusogenic replication niche called the parasitophorous vacuole
102 (PV), which is separated from the host cell cytoplasm by the PV membrane (PVM). Once the PV is formed,
103 *Toxoplasma* constitutively secretes proteins from its unique organelle, dense granules, into the PV lumen
104 (19). Many dense granule proteins (GRAs) are parasite effector proteins, which are associated with the
105 PVM and involved in organizing the structure and environment of the PV (20), nutrient acquisition (21, 22),
106 and modulation of host immune responses (23–26). Although most GRAs contribute to *Toxoplasma* fitness
107 inside host cells, some of the effector proteins on the PVM can also trigger the host immunity against the
108 parasite. For example, PVM-localized *Toxoplasma* effector GRA15 can recruit ubiquitin ligase TRAF6 to
109 the PVM, leading to lysosomal degradation of the PV in interferon-gamma (IFN γ)-activated human
110 fibroblast (27). Our previous study also identified three PVM-localized GRAs that are required for NLRP1
111 inflammasome activation in the LEW rat macrophages (28). Given that these GRA effectors were identified
112 in single parasite clones generated from a chemical mutagenesis screen, it remains unclear whether other
113 *Toxoplasma* effectors can trigger NLRP1 inflammasome activation as well as the mechanism involved in
114 the inflammasome activation.

118 RESULTS:

119 Genome-wide CRISPR screens identify *Toxoplasma* effectors that activate NLRP1 inflammasome in 120 LEW rat macrophages.

121 To ensure that we did not miss additional parasite effectors involved in NLRP1 inflammasome activation,
122 we performed a genome-wide CRISPR screen in *Toxoplasma* followed by infection of LEW rat bone
123 marrow-derived macrophages (BMDMs) (**Fig 1A**). In two independent screens, we observed a decrease in
124 cell death of LEW rat BMDMs (**Fig 1B**) and an increase in the number of parasites that could replicate
125 within the BMDMs (**Fig 1C**), indicating that mutant parasites that failed to activate the NLRP1
126 inflammasome were enriched in the population. Further selections of the parasite population allowed us to
127 enrich for these mutant parasites, resulting in a final population where > 60% of LEW rat BMDMs were
128 viable after infection (**Fig 1D**) and ~ 80% of the parasites were able to replicate within the macrophages
129 (**Fig 1C**). Using Illumina sequencing, we determined the abundance of single guide RNAs (sgRNAs)
130 present in these parasite populations to identify enriched sgRNAs that targeted genes responsible for
131 activating the NLRP1 inflammasome. Consistent with our previous chemical mutagenesis screen (28), our
132 CRISPR screen enriched for only three signal peptide-coding genes, which encoded dense granule
133 proteins GRA35, GRA42, and GRA43 (**Fig 1D** and **Table S1**). This strongly suggests that these three
134 GRAs are the only *Toxoplasma* secreted proteins responsible for inducing pyroptosis in LEW rat BMDMs.
135 Since we previously showed that GRA42 and GRA43 localize inside the PV lumen and facilitate the correct
136 localization of GRA35 to the PVM (28), we focused on GRA35 hereafter to understand how this PVM-
137 localized effector triggers NLRP1 inflammasome activation.

139 GRA35 interacts with host E3 ubiquitin ligase ITCH.

140 GRA35 is not predicted to have proteolytic or ubiquitin ligase activity. Therefore, to gain insight into the
141 molecular mechanism underlying GRA35-mediated NLRP1 inflammasome activation, we first determined
142 the topology of GRA35 on the PVM. GRA35 has a single transmembrane domain that separates the protein
143 into a short N-terminus (97 amino acids) and a long C-terminus (237 amino acids). To clarify its topology,
144 we selectively permeabilized the host plasma membrane, but not the PVM, using 0.001% Digitonin in host
145 cells infected with a parasite strain expressing GRA35 C-terminally tagged with the HA epitope (**Fig 2A**).
146 We used SAG1 antibodies, which stain the parasite plasma membrane, to identify fully permeabilized

147 vacuoles in infected cells. In host cells containing SAG1-negative vacuoles, we observed HA antibody
148 staining on the PVM (**Fig 2A**), indicating that GRA35 was localized on the PVM with its C-terminus facing
149 the host cytosol.

150 The C-terminus of GRA35 contains several coiled-coil domains (28), which are often involved in
151 protein-protein interactions. However, we previously did not observe a direct interaction between GRA35
152 and LEW rat NLRP1 (28), indicating that *Toxoplasma* GRA35 likely induces NLRP1 inflammasome
153 activation by interacting with other host proteins. To identify GRA35 interaction partners, we
154 immunoprecipitated GRA35 from *Toxoplasma*-infected rat BMDMs. Mass spectrometry analysis identified
155 only one rat protein, the E3 ubiquitin ligase ITCH, that was specifically and consistently present in GRA35
156 immunoprecipitated samples (**Fig 2B**). To confirm the direct interaction between GRA35 and rat ITCH, we
157 performed coimmunoprecipitation experiments in HEK293T cells expressing FLAG-tagged rat ITCH (FLAG-
158 rITCH) and HA-tagged GRA35 or HA-tagged control dense granule proteins (e.g., GRA42, GRA43, and
159 GRA6) (**Fig 2C**). We observed that GRA35, but not the control dense granule proteins, specifically
160 immunoprecipitated rat ITCH (**Fig 2C**). Furthermore, we found that the C-terminus (amino acid 142 to 378)
161 of GRA35 had a stronger affinity for rat ITCH than full-length GRA35 (**Fig 2D**), indicating that the C-
162 terminus serves as the functional domain of GRA35 for interacting with host proteins. To confirm the direct
163 interaction between rat ITCH and *Toxoplasma* GRA35, we performed reverse immunoprecipitations and
164 found that rat ITCH binds specifically to the C-terminus of GRA35 (**Fig 2E**). ITCH is a member of the
165 NEDD4 family of E3 ubiquitin ligases and has several domains, including an N-terminal Ca²⁺-dependent
166 phospholipid-binding C2 domain, four tandem WW domains for substrate binding, and the C-terminal HECT
167 domain for interaction with an E2 ubiquitin transferase, leading to the ubiquitination of substrates(29). To
168 determine the domain of ITCH that binds to *Toxoplasma* GRA35, we generated FLAG-tagged constructs
169 expressing different ITCH truncations (**Fig 2F**). By performing coimmunoprecipitations with the GRA35 C-
170 terminus, we found that ITCH only binds to GRA35 in the presence of its N-terminal C2 domain (**Fig 2G**).
171 Given that the C2 domain of NEDD4 family E3 ubiquitin ligases mainly involves binding to membranes (30,
172 31), we determined the localization of ITCH in *Toxoplasma*-infected LEW rat BMDMs (**Fig 2H**). We found
173 that ITCH is recruited to the PVM of ~10% of wild-type parasites, whereas ITCH PVM coating is completely
174 absent in BMDMs infected with $\Delta gra35$ parasites (**Fig 2H**). Taken together, these results demonstrate that

175 the *Toxoplasma* effector GRA35 localizes on the PVM where its host-cytosol facing C-terminus recruits the
176 host E3 ubiquitin ligase ITCH.

177
178 **ITCH mediates NLRP1 inflammasome activation triggered by *Toxoplasma* infection in LEW rat**
179 **macrophages.**

180 As a HECT-type E3 ubiquitin ligase, ITCH primarily targets substrates for K48-linked ubiquitination, which is
181 a well-established signal for canonical proteasomal degradation (32). Consistent with the observation that
182 blocking proteasome activity prevents NLRP1 inflammasome activation (6, 33, 34), we found that
183 *Toxoplasma* was unable to induce cell death in LEW rat BMDMs in the presence of the proteasome
184 inhibitor MG132 (**Fig 3A**). MG132 treatment did not block parasite invasion (**Fig S1**), suggesting that
185 *Toxoplasma*-induced LEW rat NLRP1 inflammasome activation is also mediated by the “functional
186 degradation” of the repressive NLRP1 N-terminus. To further understand the role of ITCH in mediating
187 NLRP1 inflammasome activation in LEW rat BMDMs after *Toxoplasma* infection, we generated *Itch*
188 knockout BMDMs by delivering recombinant Cas9 protein in a complex with three sgRNAs targeting the
189 second exon (only ~70bp in the first exon) of rat *Itch* (**Fig 3B**). PCR amplification of a region containing the
190 *Itch* sgRNA targeting sites resulted in reduced band size compared to negative control cells (**Fig 3C**), and
191 Sanger sequencing confirmed that the *Itch* locus is disrupted at the sgRNA targeting sites (**Fig S2**).
192 Inference of CRISPR Edits (ICE) analysis of the sequencing products revealed that 86% ± 4.1% of cells (n
193 = 4) had CRISPR editing in the *Itch* locus around the sgRNA targeting sites (**Fig 3D**). As a positive control,
194 we also generated *Nlrp1* knockout LEW rat BMDMs using a similar approach, which resulted in 96% ± 1.9%
195 (n = 4) editing efficiency in the *Nlrp1* locus (**Fig 3D**). Significantly less cell death was observed in BMDMs
196 transfected with *Itch* or *Nlrp1* sgRNAs after infection with *Toxoplasma* compared to BMDMs transfected
197 with Cas9 protein alone or Cas9 protein with scrambled sgRNAs (targeting *E. coli* LacZ) (**Fig 3E**).
198 Additionally, there was increased parasite replication in BMDMs transfected with *Itch* or *Nlrp1* sgRNAs,
199 indicated by a significantly higher proportion of vacuoles containing more than 1 parasite compared to
200 control BMDMs (**Fig 3E**). Taken together, these results reveal that the host E3 ubiquitin ligase ITCH plays
201 an important role in the NLRP1-mediated cell death upon *Toxoplasma* infection of LEW rat BMDMs.

202 To determine if ITCH directly ubiquitinates the LEW rat NLRP1, we performed an *in vitro*
203 ubiquitination assay using recombinant human ITCH protein (GRA35 also interacts with human ITCH as

204 shown in **Fig 4A**), which shares over 90% homology with rat ITCH, and FLAG-tagged LEW rat NLRP1
205 protein produced from SF9 insect cells (**Fig S3A**). However, we only observed a strong auto-ubiquitination
206 of ITCH in the presence of ATP and did not detect any ubiquitination of NLRP1. While this assay allowed
207 us to assess the direct ubiquitination of NLRP1 by ITCH *in vitro*, it remained unclear whether *Toxoplasma*
208 infection triggers the ubiquitination activity of ITCH, particularly in the context of ITCH interacting with
209 GRA35. To address this question, we performed an in-cell ubiquitination assay in HEK293T cells that stably
210 expressed LEW rat NLRP1 fused with EGFP at its N-terminus and tagged with the MYC epitope at its C-
211 terminus. We co-expressed HA-tagged ubiquitin and FLAG-tagged ITCH into the cells and then performed
212 GFP-immunoprecipitation to capture the NLRP1 protein. However, we did not observe any ubiquitination of
213 NLRP1 regardless of *Toxoplasma* infection (**Fig S3B**). We also found that ITCH did not directly interact with
214 rat NLRP1 in the same experiment (**Fig S3B**).

215 Previous studies have shown that the host serine dipeptidase DPP9 can inhibit NLRP1
216 inflammasome activation by sequestering the free NLRP1 C-terminus and thereby blocking NLRP1
217 assembly with Caspase-1 (35, 36). The inhibition of DPP9 with a small molecule called Val-boroPro (VbP)
218 causes NLRP1 inflammasome activation in various cell types (33, 34, 37). VbP treatment specifically
219 induced cell death in BMDMs isolated from LEW rats but not Brown Norway (BN) rats, which have an
220 NLRP1 inflammasome that cannot recognize *Toxoplasma* infection (**Fig S4A**). This suggests that
221 *Toxoplasma* may activate the NLRP1 inflammasome via acting on DPP9. VbP treatment induced cell death
222 in control and *Itch* knockout rat BMDMs but not in *Nlrp1*-knockout BMDMs (**Fig S4B**). To investigate
223 whether ITCH acts upstream of DPP9 to activate the NLRP1 inflammasome, we determined if ITCH
224 interacts with DPP9 and mediates its ubiquitination. However, we found that ITCH neither directly
225 ubiquitinates DPP9 *in vitro* (**Fig S3A**) nor interacts with DPP9 (**Fig S4C**). Surprisingly, our previous
226 transcriptomic analysis (15) of the BMDMs isolated from various rat strains indicated that DPP9 is not even
227 expressed in rat macrophages (**Fig S4D**). Collectively, these results suggest that ITCH mediates NLRP1
228 inflammasome activation in *Toxoplasma*-infected LEW rat macrophages using a yet-to-be-determined
229 mechanism that is independent of NLRP1 ubiquitination and DPP9 inhibition.

230
231 **ITCH-GRA35 interaction affects *Toxoplasma* susceptibility to IFN γ -induced growth inhibition in**
232 **human fibroblasts.**

233 ITCH plays a vital role in immune-related functions, such as T-cell responses and apoptosis, across a
234 range of host species (38). Moreover, our previous proteomic analysis in human foreskin fibroblasts (HFFs)
235 (27) indicated that GRA35 also interacts with human ITCH in the presence or absence of IFN γ activation
236 (**Fig 4A** and **Table S3**). Therefore, we sought to determine whether GRA35-ITCH interaction triggers host
237 mechanisms response to *Toxoplasma* infection other than inflammasome activation in rat macrophages.
238 Firstly, we investigated whether GRA35 plays a role in pyroptotic cell death in IFN γ -activated HFFs. To do
239 so, we infected both naive and IFN γ -activated HFFs with wild-type, GRA35-complemented, and $\Delta gra35$
240 parasites. We found that the levels of LDH release, a marker of host cell death, were similar between HFFs
241 infected with wild-type, $\Delta gra35$, and GRA35-complemented parasites (**Fig 4B**). While the overall levels of
242 cell death did not change significantly, the absence of GRA35 led to significantly lower growth inhibition in
243 IFN γ -activated HFFs (**Fig 4C**). This indicates that GRA35 is involved in determining *Toxoplasma*
244 susceptibility to IFN γ -mediated restriction in HFFs.

245 To determine whether ITCH is involved in IFN γ -induced parasite inhibition, we used the
246 CRISPR/Cas9 technique to generate pooled *Itch*-knockout HFFs (**Fig 4D**). ITCH knockout did not affect the
247 growth of wild-type parasites in the presence of IFN γ but caused $\Delta gra35$ parasites to become significantly
248 more resistant to IFN γ -induced growth inhibition in ITCH knockout HFFs (**Fig 4E**). This result indicates that
249 ITCH contributes to IFN γ -mediated parasite restriction. When cells are activated by IFN γ , the pathogen-
250 containing vacuole membrane of several intracellular pathogens, including *Toxoplasma*, can be marked
251 with polyubiquitin chains, which initiates a cascade of molecular events leading to vacuole disruption (39).
252 To understand how the interaction between GRA35 and ITCH affects IFN γ -induced parasite growth
253 inhibition, we examined the recruitment of ITCH (**Fig 4F**) and ubiquitin (**Fig 4G**) to the PVM in control
254 (scrambled sgRNA-transduced) and *Itch*-knockout HFFs. We found that 50~60% of vacuoles of wild-type or
255 GRA35-complemented parasites were coated with ITCH in control HFFs, regardless of IFN γ activation,
256 whereas $\Delta gra35$ parasites had significantly lower ITCH recruitment (**Fig 4F**). However, a significantly lower
257 level of ubiquitin coating was observed in *Itch*-knockout HFFs in the presence of IFN γ compared to without
258 IFN γ activation regardless of GRA35 expression (**Fig 4G**). These results indicate that ITCH does not
259 exclusively mediate the ubiquitination of the vacuole in HFFs and may affect the dynamics of other host E3
260 ubiquitin ligases that mark the PVM with ubiquitin. RNF213, another host E3 ubiquitin ligase that is an
261 IFN γ -stimulated gene and actively accumulates on the vacuole, mediates *Toxoplasma* growth inhibition (40,

41). We found that $\Delta gra35$ parasites had significantly less RNF213 on the PVM, especially in IFN γ -activated *Itch*-knockout HFFs, compared to wild-type and $\Delta gra35 + GRA35HA$ parasites (**Fig 4H**). Taken together, these results indicate that the interaction between ITCH and GRA35 plays a role in determining parasite fitness in human cells by affecting the RNF213 loading and ubiquitin status on the vacuole.

DISCUSSION:

The intricate interplay involving the *Toxoplasma* infection and the activation of effector-triggered immunity provides profound insight into the complexity of host-pathogen interactions and the robustness of the mammalian immune system. This study demonstrates that the *Toxoplasma* secreted effector GRA35 can interact with the host E3 ubiquitin ligase ITCH, which recruits this ubiquitin ligase to the parasite's replication niche. E3 ubiquitin ligases are a group of critical enzymes in the host cell's ubiquitination system, contributing significantly to various cellular processes, including protein degradation and turnover, cell cycle progression, and signal transduction (42). Besides maintaining the homeostasis of host cells, E3 ubiquitin ligases also participate in host immune responses. Particularly, our results reveal that E3 ubiquitin ligase ITCH mediates the recognition of *Toxoplasma* infection via its interaction with GRA35, which leads to NLRP1 inflammasome activation in rat macrophages and cell-autonomous response in human fibroblast activated by IFN γ . Eventually, both pathways contribute to parasite restriction in the host cells, highlighting the novel role of E3 ubiquitin ligase ITCH in mediating effector-triggered immunity and acting as a sentinel against pathogen infection across host species and cell types.

Although the exact molecular mechanism of GRA35-ITCH interaction-induced inflammasome activation is still unclear, inhibition of proteasome activity blocks pyroptosis in *Toxoplasma*-infected macrophages in a manner consistent with the "functional degradation" model for NLRP1 activation. However, we found that ITCH does not directly ubiquitinate or interact with LEW rat NLRP1, suggesting that ITCH may act on another host protein to initiate the "functional degradation" of NLRP1. Given that ITCH is not involved in DPP9 inhibition, another E3 ubiquitin ligase may be the potential host protein that is directly involved in NLRP1 inflammasome activation. It was also intriguing to discover that DPP9 is not expressed in LEW rat macrophages, but VbP still efficiently causes NLRP1 inflammasome activation. This implies that VbP not only targets DPP9 but also other host proteins that could maintain the homeostasis of the NLRP1 inflammasome. On the other hand, our results suggest that the mechanism of NLRP1

291 inflammasome activation induced by VbP and *Toxoplasma* is different, and that the interaction between
292 ITCH and GRA35 on the PVM is a unique mechanism for the host to recognize *Toxoplasma* infection. Our
293 data indicate that ITCH interacts with GRA35 via its C2 domain, which is known for membrane binding as
294 well as mediating protein oligomerization (43). Thus, one possibility is that GRA35 could mediate ITCH
295 oligomerization, which further enhances the ubiquitin ligase activity (44) and promotes its binding to the
296 substrates, potentially including proteins that modulate LEW rat NLRP1 stability. Alternatively, this binding
297 event could initiate broader perturbations to host cell homeostasis that are ultimately integrated through the
298 inflammasome response.

299 In previous proteomic studies, ITCH was also identified as a host protein that is enriched on the
300 vacuole in parasite-infected HFFs (45, 46). Our study shows that in addition to its role in mediating NLRP1
301 inflammasome activation in LEW rat macrophages, ITCH also contributes to IFN γ -mediated parasite
302 restriction in human fibroblasts. When human and murine cells are activated by IFN γ , vacuole ubiquitination
303 occurs, leading to vacuole disruption and parasite inhibition (39). However, only a few host E3 ubiquitin
304 ligases have been found to target the vacuole and participate in its ubiquitination. Although ITCH
305 recruitment to the vacuole is solely mediated by GRA35 and is not induced by IFN γ in HFFs, the deletion of
306 ITCH affects the loading of ubiquitin and the newly identified parasite restriction factor, RNF213, to the
307 vacuole. As a result, less growth inhibition was observed for $\Delta gra35$ parasites in *Itch* knockout HFFs (**Fig**
308 **4E**). Notably, the loading of RNF213 on the vacuole of wild-type parasites is also decreased in *Itch*-
309 knockout HFFs, but parasite growth inhibition remains similar to control HFFs, indicating that other host
310 IFN γ -stimulated genes are involved in this process and may target the PVM in a GRA35 dependent manner.
311 We also found that ITCH coating in $\Delta gra35$ parasites was completely abolished in LEW rat macrophages
312 (**Fig 2H**), but not in HFFs (**Fig 4F**), suggesting that other *Toxoplasma* proteins localized on the PVM
313 particularly involved in the recruitment of human ITCH to the PVM. Further studies are required to
314 determine the exact mechanism underlying ITCH-mediated parasite growth inhibition and explore the
315 interactions of other parasite proteins with ITCH from different host origins.

316 *Toxoplasma* has long been regarded as a successful and persistent intracellular pathogen, owing to
317 its capacity to establish lifelong chronic infection in a wide range of warm-blooded animals. However,
318 carrying effector proteins like GRA35, which can be recognized by the host innate immune machinery,
319 appears counterproductive to *Toxoplasma*'s survival and proliferation in the host cells. This seeming

320 contradiction highlights the intricate dynamics of host-pathogen interactions. Many effector proteins
321 released by *Toxoplasma* during host infection play critical roles in mediating the evasion of the innate
322 immune response and determining parasite virulence (47, 48). Nevertheless, a few “detrimental”
323 *Toxoplasma* effectors could temper the host's immune response to prevent parasite overgrowing, which
324 might otherwise lead to host death, an unfavorable outcome for the parasite given it relies on the host for its
325 propagation. Therefore, the detriment effector-triggered immunity poses to the parasite's immediate
326 survival could serve a greater role in ensuring long-term persistence and transmission. It is worth noticing
327 that GRA35 might also play a role in enhancing parasite fitness in other contexts. For instance, GRA35 was
328 recently identified as the top hit that maintains the growth and proliferation of type II *Toxoplasma* PRU
329 strain in IFN γ -activated human fibroblasts (49), which is different from the “detrimental” role of GRA35
330 played in the type I RH parasites demonstrated in our current study. Given that the C-terminus of GRA35
331 (the part facing to host cytosol) has a high rate of nonsynonymous/synonymous (NS/S) polymorphisms
332 among 64 different *Toxoplasma* strains (28), GRA35 has likely undergone positive selection due to the host
333 immune pressure. Since ITCH interacts with GRA35 via its C-terminus, it would be interesting to know if the
334 polymorphisms of GRA35 C-terminus affect the efficiency of ITCH recognition and, furthermore, influence
335 the outcome of *Toxoplasma* infection.

336 Although more research is needed to fully understand the mechanistic details, host E3 ubiquitin
337 ligases clearly represent a critical checkpoint in anti-*Toxoplasma* cell-autonomous response. In addition to
338 ITCH and RNF213, other host E3 ubiquitin ligases were discovered to be involved in *Toxoplasma*
339 restriction, such as TRIM21 (50) and TRAF6 (27). Addressing the precise mechanisms through which these
340 enzymes restrict *Toxoplasma* infection could pave the way for developing innovative strategies to combat
341 toxoplasmosis and provide novel insight into our understanding of effector-triggered immunity against other
342 intracellular pathogens from the perspective of host-pathogen interaction.

344 **METHODS & MATERIALS:**

345 **Reagents and antibodies**

346 Dextran sulfate sodium salt was purchased from Santa Cruz Biotechnology (Cat# sc-203917).
347 CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (MTS reagent) was obtained from Promega
348 (Cat# G3580). Proteasome inhibitor MG132 (Cat# S2619) and Caspase-1/11 inhibitor VX-765 (Cat# S2228)

349 were purchased from Selleck Chemicals. Digitonin (Cat# 300410) and Val-boroPro (Cat# 5314650001)
350 were obtained from Sigma-Aldrich. Halt™ protease and phosphatase inhibitor cocktail (Cat # 78444) was
351 purchased from Thermo Scientific.

352 Pierce™ anti-HA magnetic beads (Cat# 88837) were purchased from Thermo Scientific. Rat
353 monoclonal anti-HA (3F10) antibodies (Cat# 11867431001), Mouse monoclonal anti-FLAG (M2) antibodies
354 (Cat# F3165), and Horseradish peroxidase (HRP)-conjugated Mouse monoclonal anti-FLAG antibodies
355 (Cat# A8592) were purchased from Sigma-Aldrich. Rabbit monoclonal anti-FLAG (D6W5B) antibodies (Cat
356 # 14793S) and Mouse monoclonal anti-MYC (9B11) antibodies (Cat# 2276S) were obtained from Cell
357 Signaling Technology. Mouse polyclonal anti-V5 antibodies were purchased from MBL Life Science (Cat#
358 PM003). Rabbit polyclonal anti-GFP antibodies were purchased from Novus Biologicals (Cat# NB600-308).
359 Rabbit monoclonal anti-ITCH (D8Q6D) antibodies used for immunoblotting were purchased from Cell
360 Signaling Technology (Cat# 12117S), and purified Mouse anti-ITCH antibodies used for
361 immunofluorescence assay were purchased from BD Biosciences (Cat# 611198). Mouse monoclonal anti-
362 Ubiquitin antibodies used for immunoblotting were obtained from Santa Cruz Biotechnology (Cat# sc-8017),
363 and Mouse monoclonal anti-Ubiquitin antibodies used for immunofluorescence assay were purchased from
364 Enzo Life Sciences (Cat# ENZ-ABS840-0100). Rabbit polyclonal anti-RNF213 antibodies were purchased
365 from Sigma-Aldrich (Cat# HPA003347). Mouse monoclonal anti-SAG1 (clone DG52) antibodies were
366 described in(51). Rabbit polyclonal anti-GRA7(52) and anti-SAG1 antibodies were kindly provided by Dr.
367 John C. Boothroyd. HRP-conjugated Goat anti-Mouse/Rabbit/Rat IgG secondary antibodies were
368 purchased from Jackson ImmunoResearch Laboratories Inc. (Cat# 111-035-003/112-035-003/115-035-
369 003). HRP-conjugated Mouse anti-Goat IgG secondary antibodies were purchased from Santa Cruz
370 Biotechnology (Cat# sc-2354). Goat anti-Mouse IgG (Alexa Fluor 448/594, Cat# A11029/A11032), Goat
371 anti-Rat IgG (Alexa Fluor 594, Cat#A11007), and Goat anti-Rabbit (Alexa Fluor 488/594,
372 Cat#A11008/A11037) secondary antibodies were purchased from Thermo Scientific.

373 **Animals**

374 Six to eight-week-old female LEW rats (LEW/Crl; Strain Code: 004) and BN rats (BN/Crl; Strain
375 Code: 091) were purchased from Charles River Laboratories. The rats were housed under pathogen-
376 specific free conditions at the University of California, Davis animal facility and were allowed to acclimatize
377

378 in the vivarium for at least a week undisturbed. In the facility, rats were housed in ventilated cages on corn
379 bedding and provided with water and chow ad libitum. Cages were all on one rack at a housing density of
380 three rats per cage. The rat housing room was on a 12-hours light/12-hours dark cycle with the temperature
381 maintained at 22-25°C and the humidity range of 30-70%. The rats were monitored twice daily by
382 veterinarians, and cage bedding was changed every two weeks. All animal experiments were performed in
383 strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the
384 National Institutes of Health and the Animal Welfare Act, approved by the Institutional Animal Care and Use
385 Committee at the University of California, Davis (Assurance Number: A-3433-01).

386 387 **Culture of cells and parasites**

388 Human foreskin fibroblasts (HFFs, gift from Dr. John C. Boothroyd) were cultured in Dulbecco's
389 modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL
390 penicillin/streptomycin, and 10 µg/mL gentamicin. Primary rat bone marrow-derived macrophages (BMDMs)
391 were obtained by differentiating and cultivating bone marrow cells isolated from the tibia and femur of LEW
392 rats or BN rats in Macrophage Differentiation Media (DMEM containing 20% FBS, 2 mM L-glutamine, 10
393 mM HEPES, 1 x non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, 10
394 µg/mL gentamicin, and 30% L929 conditioned medium) for 7 days. Fully differentiated rat BMDMs used for
395 parasite infection or other experiments were cultured in Complete Macrophage Media (DMEM containing
396 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 x non-essential amino acids, 1 mM sodium pyruvate, 100
397 U/mL penicillin/streptomycin, 10 µg/mL gentamicin, and 30% L929 conditioned medium). Lenti-X cells and
398 HEK293T cells were DMEM containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 x non-essential
399 amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, and 10 µg/mL gentamicin.

400 SF9 insect cells were maintained in HyClone SFX-Insect Cell Media (Cytiva) supplemented with 1X
401 antibiotic-antimycotic (Thermo Scientific, Cat# 15240096) at 27°C with constant shaking at 100 RPM. SF9
402 cells were recently purchased from the manufacturer and were not authenticated, and these cells were not
403 regularly tested for mycoplasma contamination.

404 *Toxoplasma gondii* strains RH-Cas9(53), RH Δ ku80 Δ hxgprt(54), RH Δ hxgprt(28), RH Δ gra35(28),
405 RH Δ gra35 + GRA35HA(28), RH Δ ku80 Δ hxgprt-GRA23-HA-FLAG::DHFR(22), and RH-GRA15_{II}-HA(25)

406 were routinely passaged *in vitro* on monolayers of HFFs at 37 °C in 5% CO₂. All cells and parasite strains
407 were tested negative for mycoplasma contamination by PCR.

409 Plasmid construction

410 All the plasmids and primers used in this study are listed in Table S2. The plasmid for making the
411 GRA35 endogenously HA-tagged *Toxoplasma* strain was generated by amplifying and inserting ~1.6 kb
412 upstream of the stop codon of GRA35 into pLIC-3xHA::DHFR vector using ligation-independent cloning
413 (54). To ectopically express C-terminal HA-tagged GRA6, GRA42, or GRA43 in mammalian cells, the
414 coding sequence of mature GRA6, GRA42, or GRA43 (without signal peptide) was PCR amplified using
415 primers listed in Table S2 and flanked with the HA epitope coding sequence before the stop codon followed
416 by cloning into pcDNA3.1+ (Thermo Scientific, Cat# V79020) between *KpnI* and *EcoRI* sites. The
417 mammalian expression vector containing C-terminal HA-tagged GRA35 without signal peptide named
418 pcDNA3.1-GRA35-HA was constructed in our previous study(28). To construct the plasmid expressing the
419 GRA35 C-terminus (after the transmembrane domain), the coding sequence of GRA35^{142aa-378aa} was PCR
420 amplified using primers listed in Table S2 and flanked with the HA epitope coding sequence before the stop
421 codon followed by cloning into pcDNA3.1+ between *KpnI* and *EcoRI* sites. To generate the mammalian
422 expression construct containing full-length rat ITCH, the coding sequence of rat ITCH (Accession Number:
423 XM_008762336) was amplified using primers listed in Table S2 and cloned into *SrfI* and *EcoRI*-linearized
424 pCMVtag2B vector (N-terminal FLAG epitope-containing plasmid from Agilent Technologies, Cat# 211172)
425 using Gibson Assembly (New England Biolabs, Cat# E5510S). The other pCMVtag2B vectors containing
426 different truncated versions of rat ITCH (**Fig 2F**) were generated via Q5 Site-Directed Mutagenesis Kit
427 (New England Biolabs, Cat#E0554S) by circularizing the PCR products amplified with the primers listed in
428 Table S2. The DPP9 expression vector was generated by cloning the coding sequence of full-length rat
429 DPP9 (Accession Number: NM_001305241) flanked with the N-terminal V5 epitope tag sequence into
430 pcDNA3.1+ plasmid between *KpnI* and *EcoRI* sites. To generate SF9 protein expression vectors, the
431 coding sequence of LEW rat NLRP1, BN rat NLRP1 (Accession Number: HM_060628), or rat DPP9 was
432 subcloned into pFastBac HTB with a C-terminal FLAG epitope (named His-TEV-LEW-rNLRP1-FLAG, His-
433 TEV-BN-rNLRP1-FLAG, or His-TEV-rDPP9-FLAG). To generate the Lentiviral expression construct
434 containing LEW rat NLRP1, the coding sequence of LEW rat NLRP1 (Accession Number: HM_060633)

435 was amplified and flanked with an EGFP tag at the N-terminus and an MYC epitope at the C-terminus
436 before the stop codon followed by cloning into EcoRV-linearized pLenti-CMV-Puro-DEST plasmid
437 (Addgene #17452) using Gibson Assembly.

439 **Genome-wide CRISPR/Cas9 screen in *Toxoplasma***

440 To generate a genome-wide knockout parasite population, 500 µg of Asel-linearized sgRNA library,
441 which is a mixture of pU6-DHFR plasmids containing 10 different sgRNAs against each of the 8156
442 *Toxoplasma* genes(53), were transfected into 5×10^8 of RH-Cas9 parasites (100 µg of library plasmid for
443 each 1×10^8 of parasites per transfection) followed by infection of HFFs at an MOI = 0.5. After cultivating in
444 DMEM containing 1% FBS, 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, 10 µg/mL gentamicin, and
445 40 µM chloramphenicol (CAT) (Sigma-Aldrich, Cat# C0378-5) for 24 h, the transfected parasites were
446 grown in the DMEM containing 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 1 x non-essential amino
447 acids, 1 mM sodium pyruvate, 100U/mL penicillin/streptomycin, 10 µg/mL gentamicin, 40 µM CAT, 1 µM
448 pyrimethamine, and 10 µg/mL DNase I (New England Biolabs, Cat#M0303S) for continuous passages in
449 HFFs. To screen the parasite mutants that do not activate the NLRP1 inflammasome, LEW rat BMDMs
450 were infected with the mutant pool consistent with at least 1×10^7 of parasites harvested from the 4th lytic
451 cycle (screen #1) or 1st lytic cycle (screen #2) in HFFs at an MOI = 0.2 for 2h. After washing out the
452 extracellular parasites with PBS, the medium was replaced with a medium containing 30 mg/ml dextran
453 sulfate to block the reinvasion of parasites released from pyroptotic macrophages. At 24 h post-infection,
454 extracellular parasites lysed from pyroptotic cells were removed by washing with PBS for 3 times. The
455 surviving cells containing parasite mutants unable to activate the NLRP1 inflammasome were collected and
456 seeded onto a monolayer of HFFs. To maintain the parasite mutant diversity, 10% of the parasite
457 population lysed from HFFs were passaged to the next round selection in LEW rat BMDMs. After 4 rounds
458 of selection, we extracted genomic DNA from 1×10^7 parasites and used it to amplify the sgRNAs with a
459 barcoding primer via PCR. The resulting sample was then submitted for Illumina sequencing at the
460 Genome Center of the University of California, Davis using a NextSeq (Illumina) with single-end reads
461 using primers (P150 and P151) listed in Table S2.

462 The analysis of the library screen data was performed in R (www.R-project.org) version 4.2.0, Excel
463 (Microsoft Office) version 16.72, and previously described custom software(53, 55). Briefly, the raw reads

464 for each sgRNA were determined by aligning Illumina sequencing data to the sgRNA sequences presented
465 in the library and counting the number of exact matches. To identify the genes that underwent positive
466 selection, the raw reads of each sgRNA after 4th round selection in LEW rat BMDMs were compared to the
467 library input, and the positive selection p -value of each gene was calculated using the MAGeCK
468 algorithm(56). Genes were considered as high-confident candidates if they had at least 3 positively
469 enriched sgRNAs in 4th round selection vs. library input and met a significance threshold of positive
470 selection $p < 0.05$ in both screens.

471 472 **Generation of parasite strains**

473 To generate a *Toxoplasma* strain expressing C-terminal HA-tagged GRA35, RH $\Delta ku80\Delta hxpgrt$
474 parasites were transfected with the plasmid pLIC-GRA35-3xHA-DHFR followed by selection with 3 μ M
475 pyrimethamine. After cloning by limiting dilution, the presence of *GRA35-3xHA* in the parasites was
476 determined by immunofluorescence assays.

477 478 **Generation of NLRP1-expressing HEK293T cells**

479 HEK293T cells stably expressing LEW rat NLRP1 were generated using the Lentiviral expression
480 system. Lentiviral vector pLenti-CMV-Puro-DEST containing EGFP-rNLRP1-MYC was transfected into
481 Lenti-X cells together with packaging plasmid psPAX2 (Addgene #12260) and Lentiviral envelope plasmid
482 pMD2.G (Addgene #12259) using X-tremeGENE 9 DNA transfection reagent (Sigma-Aldrich, Cat#
483 6365787001) according to the manufacturer's instructions. At 48 h post-transfection, virus-containing
484 culture supernatant was collected, followed by mixing with polybrene (Sigma-Aldrich, Cat# TR-1003-G) at a
485 final concentration of 8 μ g/mL and adding into 6-well plates containing ~50% confluent HEK293T cells.
486 After 24 h, the cells were selected with puromycin (Sigma-Aldrich, Cat# 540411) at a concentration of 10
487 μ g/mL for 72 h. After cloning by limiting dilution in 96-well plates (~ one cell per well) with puromycin
488 selection, the positive clones of NLRP1-expressing HEK293T cells were verified by the expression of GFP
489 and MYC using immunoblotting.

490 491 **CRISPR/Cas9-mediated gene deletion in primary rat BMDMs and HFFs**

492 CRISPR/Cas9 technology was used to generate an *ITCH* or *NLRP1* deletion in LEW rat BMDMs.
493 Three sgRNAs targeting the beginning of exon 2 of the rat *Itch* gene and two sgRNAs targeting the first
494 exon of the rat *Nlrp1* gene were designed using Synthego's online Knockout Guide Design tools
495 (<https://design.synthego.com/#/>) and synthesized by Synthego (Redwood City, CA) with modification of 2'-
496 O-methyl at the 3 first and last bases and 3' phosphorothioate internucleotide linkages at the first three 5'
497 and 3' terminal RNA residues with the purpose to improve the editing efficiency and minimize nucleotide
498 acid-induced innate immune response in macrophages. As a scrambled control, sgRNAs targeting *E.coli*
499 LacZ (Accession Number: NP_414878) were chosen and synthesized. All the sgRNA sequences are listed
500 in Table S2. The *S. pyogenes* Cas9-NLS purified protein was obtained from QB3 Macrolab at the University
501 of California, Berkeley. To make gene deletions, 4×10^6 Lewis rat BMDMs were electroporated with *in vitro*
502 assembled CRISPR/Cas9 ribonucleoprotein (400 pmol of Cas9-NLS protein combined with 600 pmol of
503 sgRNAs) using the Neon transfection system (Thermo Scientific, Cat #5000S) with the following program:
504 1680 Volts/ 20 ms/1 pulse. Cells electroporated with only Cas9-NLS protein without sgRNAs were used as
505 a negative control. After recovery in Complete Macrophage Media for 48 h, 1×10^5 cells were used for
506 genomic DNA isolation while other cells were seeded onto 96 well plates or coverslips (1×10^5 cells per well
507 or coverslip) for cell viability assay and parasite per vacuole counting. To check the editing efficiency, the
508 CRISPR/Cas9 editing region was amplified from the *Itch* or *Nlrp1* genomic locus using primers listed in
509 Table S2 followed by Sanger sequencing of PCR products and analysis with Inference of CRISPR Edits
510 provided by Synthego (<https://ice.synthego.com/#/>).

511 To knockout *Itch* in HFFs, ready-to-use lentivirus particles containing three unique sgRNA targeting
512 human *Itch* or viruses containing scrambled sgRNA were purchased from Applied Biological Materials Inc.
513 (Richmond, BC, Canada). These Lentiviral particles were individually mixed with 8 $\mu\text{g}/\text{mL}$ of polybrene and
514 transduced into 6-well plates containing ~50% confluent HFFs at the MOI of 5. After 24 h, the medium was
515 replaced with fresh culture medium containing 1.5 $\mu\text{g}/\text{mL}$ of puromycin and the HFFs were cultured for 5~7
516 days (with a change of puromycin medium every other day) to select the stably transduced cells. Once the
517 cells became ~80% confluent, the HFF populations were expanded in T25 culture flasks, followed by
518 checking for the knockout of *ITCH* using immunoblotting.

519

520 **Cell viability measurement using MTS and LDH assays**

521 To determine the cell viability of rat BMDMs, a previously described MTS assay was performed(28).
522 Briefly, 1×10^5 of BMDMs isolated from LEW rats or BN rats were seeded into one well of 96-well plates
523 followed by *Toxoplasma* infection at an MOI of 1 or VbP treatment at a concentration of 2 μ M. After 24 h
524 infection/treatment, cell viability was measured by adding 3 - (4,5 - dimethylthiazol - 2 - yl) - 5 - (3 -
525 carboxymethoxyphenyl) - 2 - (4 - sulfophenyl) - 2H - tetrazolium (MTS) into culture media followed by
526 reading the OD₄₉₀ value after 1.5 h. Raw absorbance of cells without infection/treatment was considered as
527 100 percent, whereas 0 was used to stand for cells treated with lysis buffer before adding MTS. The
528 percentage of viable cells was calculated by expression of relative absorbance of *Toxoplasma*-infected or
529 VbP-treated cells vs. cells without infection/treatment.

530 To measure the cell viability of HFFs, a previously described LDH assay was performed(57). Briefly,
531 2×10^4 of HFFs seeded into one well of 96-well plates were stimulated with 10U/mL human IFN γ or left
532 unstimulated for 24 h followed by the infection with different *Toxoplasma* strains at MOI = 1. At 24 h post-
533 infection, 100 μ L of culture supernatant was mixed with LDH reagent (Sigma-Aldrich, Cat# 11644793001)
534 followed by reading the OD₄₉₀ value after 20 min incubation. Raw absorbance of cells treated with 2%
535 Triton X-100 (lysis control) was considered as 100 percent of LDH release, whereas 0 was used for the
536 uninfected cells treated with lysis buffer before measuring LDH release. The percentage of LDH release in
537 parasite-infected cells was calculated using the formula: %LDH release = (OD₄₉₀ value of infected cells –
538 OD₄₉₀ value of uninfected cells)/(OD₄₉₀ value of lysis control – OD₄₉₀ value of uninfected cells).

540 **Parasite per vacuole counting**

541 To count parasites per vacuole in LEW rat BMDMs, coverslips containing 2×10^5 LEW rat BMDMs
542 were infected with 1×10^5 parasites for 30 min followed by removing the uninvaded parasites by washing 3
543 times with PBS. After 24 h infection, the cells were fixed with 4% Paraformaldehyde (PFA) for 20 min
544 followed by permeabilization/blocking with PBS containing 3% (w/v) BSA, 5% (v/v) goat serum (Thermo
545 Scientific, Cat# 16210072), and 0.1% Triton X-100 for 30 min. The parasites were detected by incubating
546 the coverslips with mouse anti-SAG1 DG52 (1:100 dilution) and rabbit anti-GRA7 (1:3000 dilution)
547 antibodies for 1 h at room temperature. After incubating with secondary antibodies Alexa Fluor 488-
548 conjugated goat anti-mouse IgG (1:3000 dilution) and Alexa Fluor 594-conjugated goat anti-rabbit IgG
549 (1:3000 dilution) together with DAPI (Sigma-Aldrich, Cat# D9542) at the final concentration of 1 μ g/mL, the

550 coverslips were mounted with Vecta-Shield mounting oil and the microscopy was performed with NIS-
551 Elements software (Nikon) and a digital camera (CoolSNAP EZ; Roper Scientific) connected to an inverted
552 fluorescence microscope (Eclipse Ti-S; Nikon). The number of parasites in at least 100 vacuoles was
553 observed, counted, and quantified.

554 555 **Invasion assay**

556 The invasion assay was performed as previously described(58) with minor modification. Briefly,
557 1×10^5 LEW rat BMDMs seeded onto coverslips were treated with 0.5 μ M MG132 or 30 mg/mL dextran
558 sulfate (as a non-invasion control) for 2 h followed by infection with 2×10^5 RH Δ *hxgprt* parasites for another
559 30 min. After washing with PBS three times, the cells were fixed with 4% PFA for 20 min at room
560 temperature and blocked with PBS containing 3% (w/v) BSA for 30 min at room temperature, and
561 extracellular parasites were stained with rabbit anti-SAG1 (1:5000 dilution in PBS containing 3% BSA) for 1
562 h at room temperature. The cells were then permeabilized in PBS containing 3% (w/v) BSA, 5% (v/v) goat
563 serum, and 0.1% Triton X-100 for 30 min at room temperature followed by incubation with mouse
564 monoclonal anti-SAG1 DG52 (1:100 dilution) for 1 h at room temperature. Alexa Fluor 594-conjugated goat
565 anti-rabbit IgG (1:3000 dilution) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:3000 dilution) were
566 used as secondary antibodies, while 1 μ g/mL of DAPI was added to the secondary antibody solution to
567 stain host nuclei. The coverslips were mounted with Vecta-Shield mounting oil, and the microscopy was
568 performed with NIS-Elements software (Nikon) and a digital camera (CoolSNAP EZ; Roper Scientific)
569 connected to an inverted fluorescence microscope (Eclipse Ti-S; Nikon). To determine the parasite
570 invasion efficiency, at least 10 random fields were observed for all samples, and the total number of
571 green/yellow parasites (intracellular + extracellular) and red parasites (extracellular) were counted and used
572 to calculate the ratio of intracellular parasites (number of green/yellow parasites subtract the number of red
573 parasites) vs. host nucleus.

574 575 **Selective permeabilization**

576 The selective permeabilization was performed as previously described(59). Briefly, HFFs grown on
577 coverslips were infected with RH Δ *ku80* Δ *hxgprt*-*GRA35*-3xHA::*DHFR* (endogenously HA-tagged *GRA35*-
578 expressing strain) for 20 h. After fixation in 4% PFA for 10 min at room temperature, the cells were

579 quenched with PBS containing 100 mM glycine for 5 min at room temperature followed by semi-
580 permeabilization with PBS containing 0.001% digitonin for 5 min at 4°C. The samples were then incubated
581 with blocking buffer (PBS containing 10% FBS) for 30 min at room temperature, probed with antibodies
582 against the HA epitope (1:500 dilution in blocking buffer) together with antibodies against SAG1 (1:5000
583 dilution in blocking buffer) for 1h at room temperature. After incubating with secondary antibodies Alexa
584 Fluor 594-conjugated goat anti-rat IgG (1:3000 dilution in blocking buffer) and Alexa Fluor 488-conjugated
585 goat anti-rabbit IgG (1:3000 dilution in blocking buffer) together with 1 µg/mL of DAPI, the coverslips were
586 mounted with Vecta-Shield mounting oil and the microscopy was performed with NIS-Elements software
587 (Nikon) and a digital camera (CoolSNAP EZ; Roper Scientific) connected to an inverted fluorescence
588 microscope (Eclipse Ti-S; Nikon).

590 **Immunofluorescence assay for detecting the recruitment of ITCH, ubiquitin, and RNF213 to the PVM**

591 Scrambled control or *Itch*-knockout HFFs grown on coverslips were stimulated with 10U/mL human
592 IFN γ or left unstimulated for 24 h, and subsequently infected with GFP-expressing *Toxoplasma* strains
593 (wild-type or Δ *gra35*) at an MOI = 1 for another 4 h. After fixation in 4% PFA for 10 min at room
594 temperature, the cells were permeabilized/blocked with PBS containing 3% (w/v) BSA, 5% (v/v) goat serum,
595 and 0.1% Triton X-100 for 30 min followed by incubating with mouse anti-ITCH (1:100 dilution), mouse anti-
596 Ubiquitin (1:250 dilution), or rabbit anti-RNF213 (1:400 dilution) for 1 h at room temperature or overnight at
597 4°C. After incubating with secondary antibodies Alexa Fluor 594-conjugated goat anti-mouse IgG or goat
598 anti-rabbit IgG (1:3000 dilution) together with 1 µg/mL of DAPI, the coverslips were mounted with Vecta-
599 Shield mounting oil and the microscopy was performed with NIS-Elements software (Nikon) and a digital
600 camera (CoolSNAP EZ; Roper Scientific) connected to an inverted fluorescence microscope (Eclipse Ti-S;
601 Nikon). To quantify the percentage of recruitment, at least 150 vacuoles were observed and counted.

603 **Immunoprecipitation**

604 To identify the host interaction partner of GRA35, two independent immunoprecipitations were
605 performed in *Toxoplasma*-infected rat BMDMs. In the first experiment, 4×10^7 of LEW rat BMDMs were
606 treated with 50 mM VX-765 (Caspase-1/11 inhibitor) for 2 h followed by infection with RH Δ *ku80* Δ *hxgprt*-
607 *GRA35-3xHA::DHFR* or RH Δ *ku80* Δ *hxgprt- GRA23-HA-FLAG::DHFR* (as a control) at an MOI of 3. After 6 h

608 infection, the cells were harvested and lysed in 1 mL of IP-lysis buffer A (125 mM Tris-Cl pH7.5, 150 mM
609 NaCl, 1% NP40) containing 1x HaltTM protease and phosphatase inhibitor and 1 mM phenylmethylsulfonyl
610 fluoride (PMSF) for 30 min on ice. The lysate was centrifuged for 30 min at 18,000 x g, 4°C, and the
611 supernatant (soluble fraction) was incubated with 20 µL of anti-HA magnetic beads for 3 h at 4°C with
612 rotation. The beads were washed and resuspended in IP-lysis buffer A followed by Mass Spectrometry
613 analysis. For the 2nd independent experiment, 2x10⁸ BMDMs isolated from LEW rats or BN rats were pre-
614 treated with 50 mM VX-765 for 2h followed by infection with RHΔ*ku80Δhxprrt- GRA35-3xHA::DHFR* or RH-
615 GRA15_{II}-HA (as a control) at an MOI of 3 for another 8 h. The cells were lysed in 4 mL of IP-lysis buffer B
616 (25 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.65% NP40) containing 1x HaltTM protease and
617 phosphatase inhibitor cocktail and 1 mM PMSF for 30 min on ice. The lysate was centrifuged for 30 min at
618 18,000 x g, 4°C, and the supernatant was incubated with 100 µL anti-HA magnetic beads at 4°C overnight
619 with rotation. After washing, the beads resuspended in IP-lysis buffer B were subjected to Mass
620 Spectrometry analysis.

621 To confirm the interaction between GRA35 and ITCH, HEK293T cells were transfected with FLAG-
622 ITCH expressing plasmid pCMVtag2B-ITCH together with GRA35-HA expressing plasmid pcDNA3.1-
623 GRA35HA at a 1:1 ration using X-tremeGENE 9 DNA transfection reagent according to the manufacturer's
624 instructions. As controls, cells were also co-transfected with pCMVtag2B-ITCH and pcDNA3.1 vector
625 containing the coding sequence of other dense granule proteins (pcDNA3.1-GRA42HA, pcDNA3.1-
626 GRA43HA, or pcDNA3.1-GRA6HA). To check the interaction between the GRA35 C-terminus and ITCH,
627 HEK293T cells were transfected with pCMVtag2B-ITCH together with pcDNA3.1 vector expressing HA-
628 tagged GRA35 C-terminus or full-length GRA35 (as a control). To determine the ITCH domain interacting
629 with *Toxoplasma* GRA35, the pcDNA3.1 vector expressing the HA-tagged GRA35 C-terminus was co-
630 transfected with pCMVtag2B containing full-length or truncated versions of ITCH into HEK293T cells. After
631 30 h, transfected cells were scraped in ice-cold PBS and lysed in IP-lysis buffer B containing 1x HaltTM
632 protease and phosphatase inhibitor cocktail and 1 mM PMSF for 30 min on ice. The lysate was centrifuged
633 for 30 mins at 18,000 x g, 4°C, and the supernatant was incubated with anti-HA magnetic beads at 4°C
634 overnight with rotation. After washing with IP-lysis buffer B for three times, proteins bound to the beads
635 were solubilized in SDS loading buffer by boiling for 5 min and examined by immunoblotting analysis. To
636 perform reciprocal IP, HEK293T cell transfected with HA-tagged GRA35 C-terminus together with

637 pCMVtag2B-ITCH or pCMVtag2B empty vector (as a control) were lysed in IP-lysis buffer B containing 1x
638 HaltTM protease and phosphatase inhibitor cocktail and 1 mM PMSF after 30 h of transfection followed by
639 immunoprecipitation with anti-FLAG Magnetic Agarose (Thermo Scientific, Cat # A36797) at 4°C overnight
640 with rotation. After washing with IP-lysis buffer B for three times, proteins bound to the agarose were
641 solubilized in SDS loading buffer by boiling for 5 min and examined by immunoblotting analysis.

642 **Mass spectrum analysis**

644 To identify proteins in GRA35-immunoprecipitated samples, the magnetic beads after
645 immunoprecipitation were sent to the Proteomics Core Facility of the University of California, Davis, for
646 mass spectrometry analysis. After overnight on-bead digestion with trypsin, the peptide extracts were
647 analyzed by LC-MS/MS using a Thermo Scientific Q Exactive Plus Orbitrap Mass Spectrometer in
648 conjunction with Thermo Scientific Proxeon Easy-nLC II HPLC and Proxeon nanospray source (for 1st
649 independent experiment) or using a Thermo Scientific Dionex UltiMate 3000 RSLC system in conjunction
650 with Thermo Scientific Orbitrap Exploris 480 instrument (for 2nd independent experiment). Mass
651 spectrometry raw files were searched using Fragpipe 16.0(60) against the UniProt *Toxoplasma gondii* and
652 rat database with default search settings. Decoy sequences were generated, appended and laboratory
653 contaminants added within Fragpipe. Decoy False Discovery Rates were controlled at 1% maximum using
654 both the Peptide and Protein prophet algorithms(61). Search results were loaded into Scaffold (version
655 Scaffold_5.0.1, Proteome Software Inc., Portland, OR) for visualization purposes. Proteins that contained
656 similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the
657 principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. The total
658 unique spectrum count for all samples is available in Table S3.

659 **Protein expression**

661 Recombinant His-TEV-rDPP9-FLAG, His-TEV-Lew-rNLRP1-FLAG, and His-TEV-BN-rNLRP1-FLAG
662 were purified similarly to human His-DPP9 (<https://www.protocols.io/groups/hao-wu-lab>). Baculoviruses
663 containing each of these proteins were prepared using the Bac-to-Bac system (Invitrogen) and used to
664 generate baculovirus-infected SF9 insect cells. To express His-TEV-rDPP9-FLAG, His-TEV-Lew-rNLRP1-
665 FLAG, or His-TEV-BN-rNLRP1-FLAG, 1 mL of the corresponding baculovirus-containing cells was used to

666 infect each L of SF9 cells. Cells were harvested 48 h after infection by centrifugation (1,682 x g, 20 min),
667 washed once with phosphate-buffered saline (PBS), flash-frozen in liquid nitrogen, and stored at -80 °C.
668 The thawed pellet from 2 L of cells was resuspended in lysis buffer (80 mL, 25 mM Tris-HCl pH 8.0, 150
669 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine abbreviated as TCEP, 5 mM imidazole), sonicated (3 s on 7
670 s off, 3.5 min total on, 50% power, Branson), and ultracentrifuged (186,000 x g, 1.5 h, 45 Ti fixed-angle
671 rotor, Beckman). After centrifugation, the supernatant was incubated with 1 mL Ni-NTA resin at 4 °C for 1 h.
672 The bound Ni-NTA beads were washed once in batch and subsequently by gravity flow using 50-100 CV
673 wash buffer (25 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM TCEP, 25 mM imidazole). The protein was
674 eluted with buffer containing 500 mM imidazole (5 mL), spin concentrated to 0.5 mL (Amicon Ultra, 50 kDa
675 MW cutoff), and further purified by size exclusion chromatography (25 mM Tris-HCl pH 7.5, 150 mM NaCl,
676 1 mM TCEP) on a Superdex 200 10/300 GL column (Cytiva). Peak fractions were pooled, aliquoted, and
677 flash-frozen in liquid nitrogen for use in ubiquitination assays (below).

678 ***In vitro* ubiquitination assays**

680 ITCH ubiquitination reactions were carried out using the manufacturer's protocol for the Human
681 ITCH/AIP4 Ubiquitin Ligase Kit (R&D Systems, K-270). 10X reaction buffer, 10X E1 enzyme, 10X E2
682 enzyme (UBE2L3), 10X ITCH E3 ligase, and 10X ubiquitin solution were combined on ice and
683 supplemented with 1 μM rDPP9-FLAG, 1 μM Lew-rNLRP1-FLAG, 1 μM BN-rNLRP1-FLAG, or no substrate
684 control (15 μL final volume per reaction). Ubiquitination was initiated with the addition of 10X Mg²⁺-ATP or
685 water (negative control) to the reaction solution. Ubiquitination reactions were incubated at 37 °C for 2 h
686 and then quenched with the addition of 4X SDS sample buffer.

687 **Immunoblotting**

689 To detect protein interaction, total proteins from the immunoprecipitated samples were loaded and
690 run onto 12% SDS-PAGE gels followed by transferring to a polyvinylidene difluoride (PVDF) membrane. To
691 check the ITCH knockout efficiency, the lysis from at least 1x10⁶ of scrambled control HFFs or *Itch*-
692 knockout HFFs (three independent clones) were loaded and run onto 12% SDS-PAGE gels followed by
693 transferring to a PVDF membrane. Membranes were blocked in 5% milk in TBS supplemented with 1%
694 Tween-20 (TBS-T) for 1 h at room temperature followed by incubation with primary antibodies diluted in the

695 blocking buffer at 4°C overnight. After incubation with HRP-conjugated secondary antibodies, the protein of
696 interest on the membranes was visualized using ProSignal® Femto ECL Reagent (Genesee Scientific,
697 Cat# 20-302), and the images were acquired using KwikQuant Imager (Kindle Biosciences, LLC).

698 For *in vitro* ubiquitination, samples were run on either 4 to 15% or 4 to 20% Mini-PROTEAN TGX™
699 Tris-Glycine gels (BioRad) for 40-60 min at 160 V. Gels were transferred to nitrocellulose with the iBlot 2
700 Transfer System (Thermo Scientific). Membranes were blocked with phosphate buffered saline with 0.05%
701 tween-20 (PBST) supplemented with 5% milk (PBST-M) for 60 min at ambient temperature, prior to
702 incubating with primary antibody (in PBST-M) overnight at 4 °C. Blots were washed 3 times with PBST prior
703 to incubating with secondary antibody (in PBST-M) for 60 min at ambient temperature. Blots were again
704 washed 3 times and subsequently imaged with a BioRad ChemiDoc using ECL Western Blotting Substrate
705 (Thermo Scientific 32106). Antibodies used include: Ubiquitin mouse monoclonal Ab (1:2000, Santa Cruz
706 Biotechnology, sc-8017), ITCH goat Ab (1:2000, R&D Systems, K-270 proprietary), anti-FLAG-HRP mouse
707 monoclonal Ab (1:5000, Sigma, A8592), anti-mouse IgG-HRP (1:10000, Cell Signalling Technology,
708 7076P2), and anti-goat IgG-HRP (1:10000, Santa Cruz Biotechnology, sc-2354).

710 **Plaque assay**

711 Scrambled control or *Itch*-knockout HFFs seeded into 24-well plates were stimulated with human
712 IFN γ (10 or 20 U/mL) or left unstimulated for 24 h followed by infection of 100 parasites into each well.
713 Infected plates were incubated for 5 days at 37°C, and the number of plaques formed by parasite infection
714 was observed and counted under the microscope using 4 x objective. To calculate the percentage of
715 plaque loss, the following formula was used: [(Number of plaques in unstimulated HFFs - Number of
716 plaques in IFN γ -stimulated HFFs)/Number of plaques in unstimulated HFFs] \times 100. All experiments were
717 performed at least 3 times with duplicate wells for each condition.

719 **Statistical analysis**

720 All statistical analyses were performed using Prism (GraphPad) version 9.5. All the data are
721 presented as mean \pm standard deviation (SD), and the exact n values are mentioned in the figure legends.
722 For all the calculations, $p < 0.05$ are considered a significant difference. To compare parasite growth of
723 Δ *gra35* vs. wild-type parasites in IFN γ -activated HFFs, paired t-test was used. For the data with more than

724 two groups with one variable, One-way ANOVA with Tukey's multiple comparisons test was used. For one
725 variable test with two groups, the two-way ANOVA with Tukey's multiple comparisons test was used.

727 **Data Availability**

728 The authors declare that all data supporting the findings of this study are available within the article
729 and Expanded View Information files, including Source Data and uncropped immunoblotting images.
730 CRISPR screen data including raw sequencing read counts are available in Table S1. Mass spectrometry
731 data are available in Table S3. All unique materials (e.g., the variety of parasite and host cell lines
732 described in this study) are available from the corresponding author (contact: Yifan Wang,
733 yifwan@med.umich.edu; Jeroen P.J. Saeij, jsaeij@ucdavis.edu) upon reasonable request.

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740 data analysis.

742 **CONFLICT OF INTERESTS**

743 Dr. Hao Wu is a co-founder of Ventus Therapeutics. The other authors declare that they have no conflict of
744 interest.

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906 **FIGURE LEGENDS:**

907 **Fig. 1 Genome-wide CRISPR screens identify *Toxoplasma* secretory effectors that induce**
908 **pyroptosis in Lewis rat bone marrow-derived macrophages.**

909 **A.** Schematic of CRISPR screen. BMDMs, bone marrow-derived macrophages.

910 **B.** Lewis rat BMDMs were infected with indicated parasites (MOI = 1) for 24 h. Macrophage viability was
911 measured via MTS assay. Data are displayed as paired plots for each individual screen.

912 **C.** Lewis rat BMDMs were infected with indicated parasites (MOI = 0.5) for 24 h. The number of parasites
913 per vacuole was quantified by microscopy. A total of 100 to 120 vacuoles were counted per experiment.
914 Data are displayed as mean + SD with two independent experiments. Significance was determined with
915 two-way ANOVA with Tukey's multiple comparisons test.

916 **D.** Top candidate genes with at least 3 enriched sgRNAs and significant enrichment (p -value < 0.05,
917 analyzed by MAGeCK algorithm) after 4th round selection in both screens. Numbers between parentheses
918 are the number of enriched sgRNAs after the 4th round of selection.

919
920 **Fig. 2 GRA35 interacts with host E3 ubiquitin ligase ITCH.**

921 **A.** HFFs were infected with parasites expressing GRA35 endogenously tagged at the C-terminus with the
922 HA epitope for 16 h. After fixation, the cells were permeabilized with 0.001% digitonin followed by staining
923 with antibodies against SAG1 and the HA epitope. The images are representative of results from 2
924 independent experiments (scale bar = 5 μ m). Arrows indicate a fully permeabilized vacuole and arrowheads
925 denote the vacuole that is not permeabilized.

926 **B.** Lysates from Lewis rat BMDMs infected with parasites expressing C-terminal HA-tagged GRA35 or
927 other PVM-localized proteins (GRA23 or GRA15, served as negative controls) were immunoprecipitated
928 using HA antibodies followed by mass spectrometry analysis. The total unique spectrum count of host E3
929 ubiquitin ligase ITCH is indicated.

930 **C.** Lysates of HEK293T cells transiently expressing FLAG-rITCH with the indicated GRA fused with HA
931 epitope were immunoprecipitated using HA antibodies followed by immunoblotting (IB) analysis with the
932 indicated antibodies. 5% of the total lysate was loaded and used as input. The images are representative of
933 results from 2 independent experiments.

- 934 **D.** HEK293T cells transiently expressing FLAG-rITCH and either full-length (FL) or the C-terminus (Ct) of
935 GRA35 fused with the HA epitope were analyzed as in (c). The images are representative of results from 2
936 independent experiments.
- 937 **E.** HEK293T cells transiently expressing FLAG-rITCH and an HA-tagged GRA35 C-terminal fragment were
938 immunoprecipitated using FLAG antibodies and analyzed as in (c). The images are representative of
939 results from 2 independent experiments.
- 940 **F.** Schematic illustration of the constructs used for the generation of full-length or truncated rat ITCH.
- 941 **G.** HEK293T cells transiently expressing a C-terminal fragment of GRA35 fused with the HA epitope and
942 FLAG-tagged rat ITCH truncations (indicated in f) were immunoprecipitated using HA antibodies and
943 analyzed as in (c). The images are representative of results from 2 independent experiments.
- 944 **H.** Lewis rat BMDMs infected with GFP-expressing *Toxoplasma* for 4h were stained with antibodies against
945 ITCH. The images are representative of results from wild-type parasite infected BMDMs (scale bar =
946 10 μ m). The percentage of vacuoles coated with ITCH was quantified as shown on the right. Data are
947 displayed as mean \pm SD with independent experiments ($n = 3$) indicated by the same color dots.
948 Significance was determined with one-way ANOVA with Tukey's multiple comparisons test. Arrowhead
949 indicates the vacuole coated with ITCH.

950

951 **Fig. 3 Knockout of *Itch* impairs *Toxoplasma*-induced NLRP1-mediated cell death of Lewis rat**
952 **BMDMs.**

- 953 **A.** Lewis rat BMDMs pre-treated with 0.5 μ M proteasome inhibitor MG132 or left untreated for 2 h were
954 infected with *Toxoplasma* parasites (MOI = 1) for 24 h. Macrophage viability was measured via MTS assay.
955 Data are displayed as mean \pm SD with independent experiments ($n = 4$) indicated by the same color dots.
956 Significance was determined with one-way ANOVA with Tukey's multiple comparisons test.
- 957 **B.** Schematic illustration of sgRNA targeting sites in the first two exons of rat *ITCH* locus. P1 and P2 are
958 the primers used for amplifying the sgRNA targeting region and verification by Sanger sequencing.
- 959 **C.** PCR amplification of the *Itch* sgRNA targeting region using P1 and P2 from Lewis rat BMDMs
960 transfected with Cas9 protein only (blank) or Cas9 protein assembled with *Itch* sgRNAs.
- 961 **D.** Lewis rat BMDMs were transfected with *in vitro* assembled CRISPR/Cas9 ribonucleoprotein containing
962 indicated sgRNAs. The editing efficiency of *Itch* or *Nlrp1* was analyzed from the Sanger sequencing

963 products (shown in c) using the Inference of CRISPR Edits (ICE) online tool
964 (<https://www.synthego.com/products/bioinformatics/crispr-analysis>). The percentage of CRISPR edited *Itch*
965 or *Nlrp1* is displayed as mean \pm SD with independent experiments ($n = 4$) indicated by the same color dots.
966 **E.** Lewis rat BMDMs generated in (d) were infected with wild-type *Toxoplasma* (MOI = 1) for 24 h.
967 Macrophage viability was measured via MTS assay. Data are displayed as mean \pm SD with independent
968 experiments ($n = 4$) indicated by the same color dots. Significance was determined with one-way ANOVA
969 with Tukey's multiple comparisons test.
970 **F.** Lewis rat BMDMs transfected with *in vitro* assembled CRISPR/Cas9 ribonucleoprotein containing
971 indicated sgRNAs were infected with wild-type *Toxoplasma* (MOI = 0.5) for 24 h. The number of parasites
972 per vacuole was quantified by microscopy. A total of 100 to 120 vacuoles were counted per experiment.
973 Data are displayed as mean + SD with 2 independent experiments. Significance was determined with two-
974 way ANOVA with Tukey's multiple comparisons test.

975

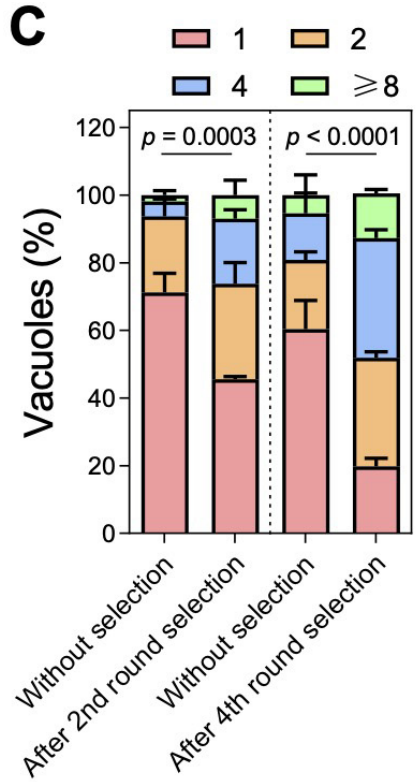
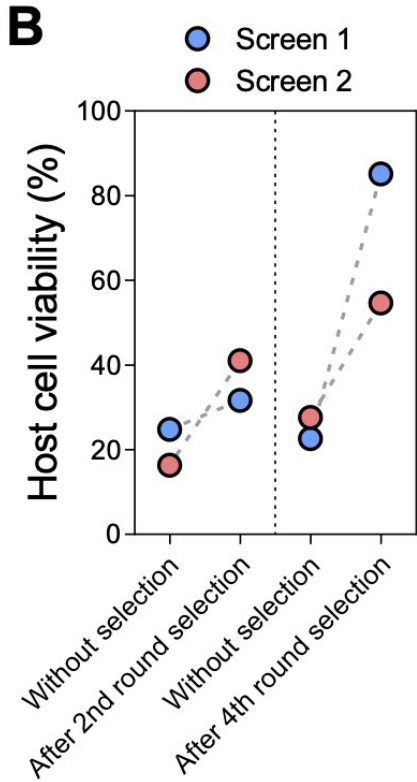
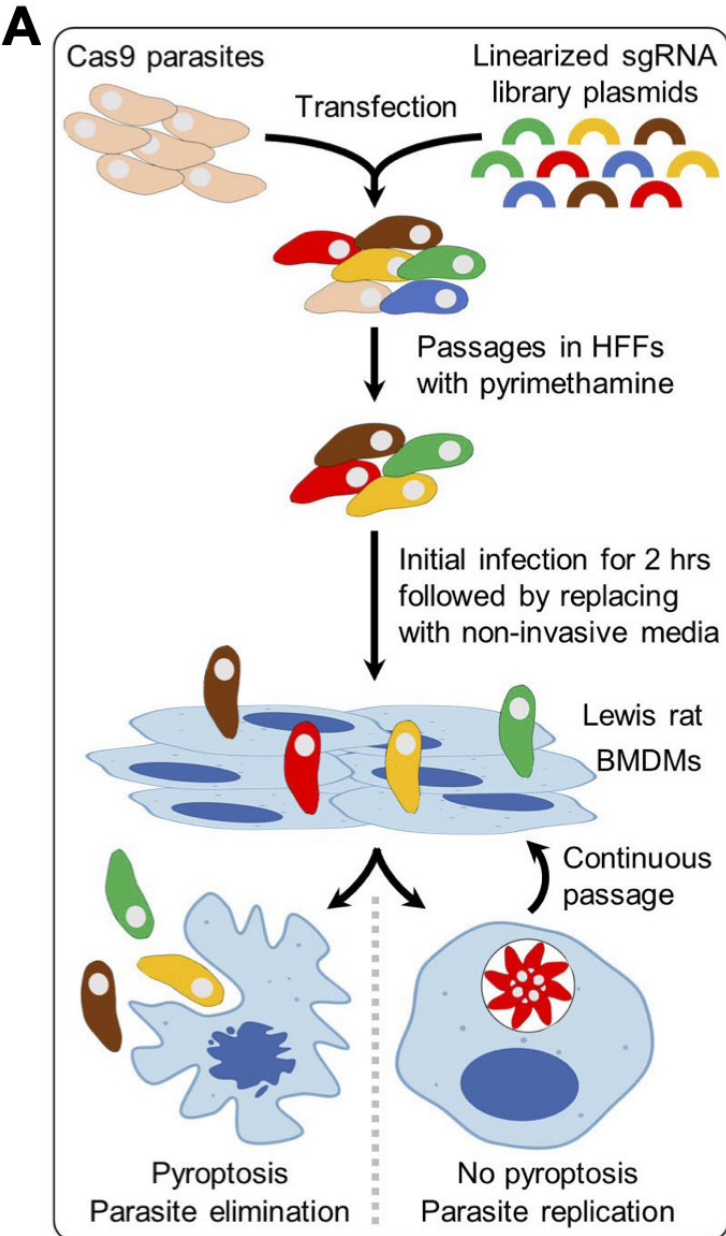
976 **Fig. 4 ITCH impacts ubiquitin and RNF213 recruitment to the PVM and alters the cell-autonomous**
977 **response to *Toxoplasma* infection in human fibroblasts.**

978 **A.** ITCH was identified as interacting with GRA35 in our immunoprecipitation coupled with mass
979 spectrometry analysis in naïve and IFN γ -activated HFFs²⁸. The total unique spectrum count of human ITCH
980 identified in GRA35-immunoprecipitated samples is shown. GRA15 serves as a PVM-localized control.
981 **B.** HFFs pre-stimulated with 10 U/ml IFN γ or unstimulated for 24 h were infected with indicated parasite
982 strains (MOI = 1) for another 24 h. Cell viability was assessed via LDH assay. Data are displayed as mean
983 \pm SD with independent experiments ($n = 3$) shown by the same color dots. Significance was determined
984 with two-way ANOVA with Tukey's multiple comparisons test.
985 **C.** HFFs pre-stimulated with 10 U/ml IFN γ or left unstimulated for 24 h were infected with indicated parasite
986 strains (100 parasites per well). Plaque number was measured at 5 days post-infection. The loss of plaques
987 in IFN γ -stimulated HFFs was calculated relative to unstimulated HFFs. Data are displayed as paired
988 scatterplots, and significance was determined with two-tailed paired *t*-test.
989 **D.** HFFs were transduced with lentivirus containing 3 individual ITCH-targeting or scrambled sgRNAs
990 followed by puromycin selection. Lysates of these HFFs were analyzed by immunoblotting with ITCH and

991 Actin antibodies. The images are representative of results from 2 independent experiments. Star (*)
992 indicates a non-specific band.

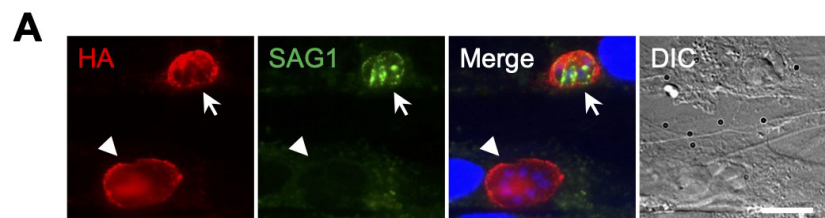
993 **E.** Indicated HFFs pre-stimulated with 10 U/ml IFN γ or left unstimulated for 24 h were infected with WT,
994 $\Delta gra35$, or $\Delta gra35 + GRA35HA$ strains (100 parasites per well). Plaque numbers were counted 5 days
995 post-infection. The loss of plaques in IFN γ -stimulated HFFs was calculated and expressed relative to
996 unstimulated HFFs. Data are displayed as mean \pm SD with independent experiments ($n = 3$) indicated by
997 the same color dots. Significance was determined with two-way ANOVA with Tukey's multiple comparisons
998 test.

999 **F-H.** Indicated HFFs pre-stimulated with 10 U/ml IFN γ or left unstimulated for 24 h were infected with
000 parasites (MOI = 1) for 4 h. The cells were fixed and stained for ITCH (**F**), Ubiquitin (**G**), or RNF213 (**H**).
001 Representative images from wild-type parasite infection in IFN γ -activated control HFFs (transduced with
002 scrambled sgRNAs) are shown (scale bar = 10 μ m). The percentage of vacuoles coated with ITCH,
003 Ubiquitin, or RNF213 was quantified. Data are displayed as mean \pm SD with independent experiments ($n =$
004 3) shown by colored dots. Significance was determined with two-way ANOVA with Tukey's multiple
005 comparisons test.



D

Gene ID	Product	<i>p</i> -value (number of enriched sgRNA)	
		Screen 1	Screen 2
TGGT1_226380	GRA35	7.3e-07 (7)	6.1e-07 (7)
TGGT1_237015	GRA43	7.3e-07 (7)	2.3e-04 (3)
TGGT1_236870	GRA42	1.4e-05 (5)	6.1e-07 (4)



B

Exp.1 Total unique spectrum count		
Rat protein	GRA35-IP	GRA23-IP
E3 ubiquitin ligase ITCH	14	0
Exp.2 Total unique spectrum count		
Rat protein	GRA35-IP	GRA15-IP
E3 ubiquitin ligase ITCH	56 (LEW) 53 (BN)	0 (both)

