

Supplementary Fig. 1 IFNy restricts parasite growth in murine BMDMs.

(a) Murine BMDMs pre-stimulated with IFN γ (1 or 100 ng/mL) or left unstimulated for 24 h were infected with luciferase-expressing type I (RH) parasites for 24 h and parasite growth was measured by luciferase assay. Parasite growth in IFN γ -activated BMDMs is expressed relative to growth in naïve BMDMs. Data are displayed as mean ± SEM with independent experiments (*n* = 3) indicated by the same color dots. The significant difference was analyzed with one-way ANOVA with Tukey's multiple comparisons test. (b) Murine BMDMs pre-stimulated with TNF α (10 ng/mL) or IFN γ (100 ng/mL) and TNF α (10 ng/mL) for 24 h or left unstimulated were infected with luciferase-expressing RH parasites for another 24 h and parasite growth in naïve BMDMs. Data are displayed as average ± SEM with independent experiments (*n* = 4) indicated by the same color dots. The significant difference was analyzed as average ± SEM with independent experiments (*n* = 4) indicated by the same color dots.



Supplementary Fig. 2 BMDM stimulation with IFNγ for 4 or 24 hours induces the expression of a similar set of genes.

(a) IFN γ -regulated genes in C57BL/6J and A/J BMDMs pre-stimulated with 100 ng/mL IFN γ were defined by a \geq 4-fold change (IFN γ -activated *vs.* naïve BMDMs) in gene expression values (FPKM). Data are displayed as a heat map of log₂ fold change of the 251 genes with either \geq 4-fold upregulated (197 genes) or \geq 4-fold downregulated (54 genes) in IFN γ -activated BMDMs (*n* = 2). The complete set of genes is listed in Supplementary Data 3.

(b) Correlation between the log₂ fold change of IFNγ-regulated genes after 4 and 24 h of IFNγ stimulation in C57BL/6J (left) and A/J (right) BMDMs. Perfect correlation is indicated by a grey diagonal dotted line.
(c) Differentially regulated pathways between IFNγ-stimulated *vs*. naïve BMDMs were identified using GSEA analysis and the MSigDB database. The 4 and 24 h time points from A/J and C57BL6/J BMDMs were treated as replicates as the goal was to identify pathways that are similarly regulated between these samples. As an example, the downregulation of the cholesterol homeostasis pathway in IFNγ-stimulated *vs*. naïve BMDMs is shown.

(**d**) An example of specific genes involved in the cholesterol homeostasis pathway (from c) regulated in IFNγ-stimulated BMDMs is presented as heat plots. Exact FPKM values of genes involved in cholesterol metabolism are in Supplementary Data 3.



Supplementary Fig. 3 related to Fig.1.

(a) Potential bottlenecks were assessed by determining the abundance disparity of control guides among different samples from each screen illustrated as Lorenz curves. These curves indicate Gini coefficients for all the samples: S1, 0.33 for library, 0.80 for early passage HFF, 0.84 for HFF control, 0.84 for Naïve BMDM, and 0.90 for IFNγ BMDM; S2, 0.35 for library, 0.76 for early passage HFF, 0.80 for HFF control, 0.80 for Naïve BMDM, and 0.81 for IFNγ BMDM; S3, 0.34 for library, 0.53 for early passage HFF, 0.77 for HFF control, 0.78 for Naïve BMDM, and 0.78 for IFNγ BMDM. Some curves are overlapped due to the similarity between Gini indices.

(b) Scatter plot with mean fitness (Naïve BMDM *vs.* HFF control) on the x-axis and $-\log_{10}(p$ -value) on yaxis. All 193 candidate genes expressed in murine macrophage⁵⁶ with p < 0.05 (dotted line) analyzed with One-sided Wilcoxon signed-rank test by comparing with control genes and Cohen's $d \ge 0.8$ are indicated as orange dots with dark orange dots indicating 9 high-confidence candidate genes (Table 1).

(c) Scatter plot with mean fitness (IFN γ *vs.* Naïve BMDM) on the x-axis and $-\log_{10}(p$ -value) on y-axis. All 160 candidate genes expressed in murine macrophage⁵⁶ with *p* < 0.05 (dotted line) analyzed with One-sided Wilcoxon signed-rank test by comparing with control genes and Cohen's *d* ≥ 0.8 are indicated as pink and red dots with red dots indicating 17 high-confidence candidate genes (Table 1).



(a) Schematic diagram depicting the genomic loci of the genes of interest (GOI) (top) and the CRISPR/Cas9-targeting site (red box). Linearized pTKO plasmid containing GFP-coding sequence and HXGPRT (HPT) selection cassette (middle) was used as a repair template to disrupt the GOI (bottom) after mycophenolic acid and xanthine selection. P1 and P2 refer to the primers used for confirming locus

Supplementary Fig. 4 Generation and confirmation of knockout and complemented parasites.

disruption. P1 + P3 or P2 + P3 are used to check insertion of the repair template into the GOI locus. (**b**) GRA45 complementation was performed by homologous recombination of *GRA45HA*-expressing cassette (middle) into *UPRT* locus (top). The coding sequence of *UPRT* was replaced with *GRA45HA* (bottom) after FUDR selection. (c) Schematic strategy used to delete the entire coding region of *GRA45* (top) by inserting DHFR* in RH $\Delta ku80$::*MYR1-3xHA* strain. Transfection of sgRNAs targeting the *GRA45* locus (red box) together with the DHFR*-expressing amplicon flanked with 60 bp of 5-'UTR and 3'-UTR of *GRA45* (middle) after pyrimethamine selection was used to generate *GRA45* deletions.

(d) *GRA45* knockout in RH-Luc+/ $\Delta hxgprt$ parasites and complementation of the gene in the *UPRT* locus.

(e) *TGGT1_263560* knockout in RH-Luc+/Δ*hxgprt* parasites.

(f) *TGGT1_269620* (top), *TGGT1_232670* (middle) and *MYR1* (bottom) knockout in RH-Luc+/Δ*hxgprt* parasites.

(g to i) Individual knockout of *GRA22* (g), *ROM1* (h) or GRA7 (i) in the RH-Cas9∆*hxgprt* background.

(j) *GRA45* knockout in RHΔ*ku80*::*MYR1-3xHA* parasites.

(**k**) Complementation of mutant versions of *GRA45* in the *UPRT* locus of Δ *gra45* parasites.

All images are representative of results from 2 independent experiments.



Supplementary Fig. 5 Parasite growth in naïve murine BMDMs and MEFs of individual gene

knockouts identified from loss-of-function screen in IFN γ -stimulated BMDM.

(**a** to **e**) Raw luciferase reads (RLU) of WT, $\Delta TGGT1_{269620}$ (a, n = 6), $\Delta TGGT1_{232670}$ (b, n = 5),

 $\Delta TGGT1_263560$ (c, *n* = 4), $\Delta gra22$ (d, *n* = 3) or $\Delta TGGT1_269950$ (e, *n* = 4) parasites in naïve murine

BMDMs were normalized to 100% viability based on their viability measured by plaque assays. Data are displayed as mean \pm SEM with independent experiments indicated by the same color dots. The significant difference between WT and knockout was analyzed with two-tailed paired *t* test.

(f) RLU of WT, $\Delta gra45$, and $\Delta gra45$ complemented with C-terminal HA tagged WT or indicated mutant version of *GRA45* parasites in naïve murine BMDMs were normalized to 100% viability based on their viability measured by plaque assays. Data are displayed as mean ± SEM with independent experiments (*n* = 3) indicated by the same color dots. Not significant (n.s.), one-way ANOVA with Tukey's multiple comparisons test.

(g) Confluent MEFs were infected with indicated parasites for 5 days. Areas of at least 40 plaques per experiment were measured. Data are displayed as mean \pm SEM with independent experiments (n = 3) indicated by the same color dots. The significant difference was analyzed with one-way ANOVA with Tukey's multiple comparisons test.

(h) Representative images of plaque size and morphology of WT, $\Delta gra22$, and $\Delta TGGT1_263560$ parasites in MEFs (scale bar = 200 µm). The images are representative of results from at least 40 plaques examined over 3 independent experiments.

(i) Intracellular parasites expressing endogenously HA-tagged *TGGT1_263560* were fixed, permeabilized, and subjected to immunofluorescent assays with anti-HA (red) and anti-GRA2 (green) antibodies (scale bar = 5 μm). The images are representative of results from 2 independent experiments.



Supplementary Fig. 6 Growth of $\Delta gra45$, $\Delta gra22$, $\Delta TGGT1_263560$, and $\Delta TGGT1_269950$ parasites in naïve rat BMDMs and THP-1 macrophages.

(**a** and **b**) Raw luciferase reads (RLU) of WT, $\Delta gra45$ (a, n = 4), $\Delta gra22$ (a, n = 4), $\Delta TGGT1_263560$ (b, n = 3) or $\Delta TGGT1_269950$ (b, n = 3) parasites in naïve Brown Norway rat BMDMs were normalized to 100% viability based on their viability measured by plaque assays. Data are displayed as mean ± SEM with independent experiments indicated by the same color dots. Not significant (n.s.), two-tailed paired *t* test. (**c** to **f**) Raw luciferase reads (RLU) of WT, $\Delta gra45$ (c, n = 7), $\Delta gra22$ (d, n = 5), $\Delta TGGT1_263560$ (e, n = 6) or $\Delta TGGT1_269950$ (f, n = 6) parasites in naïve PMA-differentiated THP-1 macrophages were normalized to 100% viability based on their viability measured by plaque assays. Data are displayed as mean ± SEM with independent experiments indicated by the same color dots. Not significant (n.s.), two-tailed paired *t* test.

a Unconserved 012345678910 Conserved 260.....27 V EQLVEREGMG 10 TGGT1_316250 FYDVE FYDVEDMFDA FYDVEDMFDA TGME49 316250 ---CSA -----RG VLAA GCLAL EQLVEREGMG TGVEG_316250 ----R G R G VE Ve HHA_316250 MFDA EQLVEKEGMG QRMENEGWN TQLVDREGMG EQMVEKDGIG QQQLDEG-FM KHLMDEENFL LLLQQQG-FL EKLLAD-GWE TGRUB 295390 OVLDWFDT NCLIV_058760 CSUI_003845 A---R VE TVEA FYDVDI IDDAVI Cyc_02015 DILKA FLE-EAH 00055290 YSFAEATAV- --GG GVGE---Y-S YDDAA VLEA ENH_00009810 SN3_01000185 YFDLW WYEVQI VLES LYEG -----AgsA Salmonella-----_____ _____ _____ ____ SFSLP HSP20_Xylella Consistency 3505434374 04640336 5564557667 3122121211 444443332 *** 60 70 80 90 320 340 FRYFTDDLAA FRYFTDDLAA FRYFTDDLAA FRYFTDDLAA FRYFTDDLAA FRYNSHDPER GAD CGGEGPH GAD CGGEGPH GAD CGGEGPH GAD CGGEGPH VNGCGAVDLQ AFRIDVKNPE AFRIDVKNPE TGGT1_316250 TGME49_316250 DKFVRKMSAM DKFVRKMSAM MSEGISMMVM MSEGISMMVM ____ GTTQV GTTQV SSPRRI DKFVRKMSAM DKFVRKMSAM GEIVAIKTTI DKFVRKMSAM GKFVRKMSAM NEWNRRKVIL QSWYRRKVII AFRIDVKNPE AFRIDVKNLE GVRVDVRELN **TGVEG 316250** SSPRRL--SEGI SMM 7 M HHA_316250 TGRUB_295390 SMM NCLIV 058760 RYLSDDPSA AAPRRL-AFRIDVKNPE CGGEGP AFRIDVKNEL SIRVDAMSSK SLRVDAQNTD SLRIDAQQKD SECGGRSPE LGCGGTEAK LGCGGKKEE KRYMTNDQNE TRVAALSPEY CSUI_003845 Cyc_02015 EAH_00055290 RVAALSPEY ARVQAISPQH -SSSSRRL NSWFRRKVIL GTPHRKMSAF -KAIEHKS---SNDVALQSM ENH 00009810 SAAA RVAAKSLET PLGCGGSSSA AYFETKAIEK JEIYQEIPES IFIPKR--AA SN3_01000185 AgsA_Salmonella QIMMEQVD KIAIESKP CGGREA HSP20_Xylella IOVGNAV 1212111111 1131223100 0000222232 0423344400 342 Consistency 4663644354 345435465 9675635 3644756656 3337763333 _ 120 LVDLGAKSM LVDLGAKSM LVDLGSKSM LVDLGSKSM LTCGCTLK LVDLGNKSM MKQWTGMVG MGQWTGMMG LYTLGSKSM MRQWTGMMG JLKRACYRMA SLF5DR-FN 734454454 . 370. 380. . . . 390. 400 150 TL TGGT1 316250 LLFF VLLD VLLD VLLD VLLD VLLE VQLE SNIP SSIP STLP YDLN GDTP ESAV 4374 YYDPQ YYDPQ YYDPQ YFDQA YHDLQ YHDLQ YWKLP YWKLP YWKLP YHRKE AA--T TAQWV 74534 G YK NDY NDY NDY D D D - GSYKALFR - GSYKALFR - GSYKALFR - GSYKALFR - SYKALFR - GTIKVLFR - GTIKVLFR - GHYKVLVK - GYYKVVIQ - GHTKVLVK N N N G N T LLFPEMRE LLFPEMRE LLFPEMRE TGME49_316250 TGVEG_316250 S<mark>VTYKRLE</mark> S<mark>VTYKRLE</mark> F TL HHA 316250 VA FSVTYKRLE ID Y ARKI DIEVTQKALE DFSVTHKRLD DFSVTSKELT DAH MDY -AE TGRUB_295390 NCLIV_058760 CSUI_003845 VMYPEMAK LLFPEMRE IMMPEMRQ D D D FRHA AERL-Cyc_02015 EAH_00055290 ENH_00009810 ATNQEIRQ ATSQEIRQ ATSEEIRQ DFTAAVYLQS DFTAAVYLQS DFAAAVYVHP DYN DLE DRN RESE-SNIEI RKDE-DTLQI KEQL<mark>G</mark>SIQQI FY AAIARO D D D AVI AQQI YFHADMRR LSALPVFA TPWPGQAA 54457766 LIN KAQKP SN3 01000185 DFAVTVKELE SPGNYK TOTY--IMTDPDEIVI 5433000323 3333154446 4334422744 AgsA_Salmonell HSP20_Xylella D 5645534344 6063574544 Consistency 441 160 LDYVESF LDYVESF LDYVESF LDYVESF IDYESF LDYIESF LGYVEF LGYVEF LGYVEF LGFVEF LGFVEF DGTFF DGTFF DGTFF DGTFF DGTFF TGGT1_316250 HVQH TAS KRKQRH KRKQRH KRKQRH NFAQKH KQKQRH KQKQRH KRPFPR GLVNLD GLVNLD GLVNLD SHVQHQ SHVQHQ SHVQHQ SHVQHQ TGME49 316250 0---TGVEG_316250 HHA_316250 _ _ **TGRUB 295390** GLIDAR YGEHE SYGEHE SHVQHQ SHTEHQ TPVHNA TPSHNA SGEYNA SGEYNA LPAQHR SV---P DNTDL -DGTHF -DGTHF -SVADI -SVADV -AAADA GLVNLD GLMDAD GISDSS NCLIV 058760 H - - -AA CSUI_003845 Cyc_02015 AAADAAH AAFDAAH GPVEVDS ---DANN K G S H Q R K R P T P R K P V Q R H EAH 00055290 s W W R ENH_00009810 SN3_01000185 EWGIL AgsA Salmonell ---- ---HSP20_Xylella Consistency 5633445 5443232<mark>5</mark>32 333<mark>6566</mark>333 343<mark>5435</mark>233 3<mark>000</mark> 043424 54455 636464 VVV 210. 220 VADEDSYWVG VADEDSYWVG VADEDSYWVG VADESYWVG VGTKDSYWEA LGRIDVHLSK LGRIDVHLSK LGRIDVHLSK LGRIDVHFSK VGRIDVHFSR TGGT1_316250 TGME49_316250 RSVSPPC RSVSPPC RSVSPPC QQVKGV CQVKGV CRSESPPC TKPTGR CTKPTGR CGAPQGR CTVDPPC KFNI KFNI KFNI RFTV KFNI RFNI FLHV VPN<mark>E</mark>QAVPL GVPNEQAVPL GVPNEQAVPL GVPNEQAVPL GVPNEQAVPL -QGREDAMTL TGVEG 316250 HHA_316250 TGRUB_295390 GEANEQPIPI G-PVNEKPVP -ITKQMMTTL -ITRQLMSTL -IAAQQLQRL -VPADEPLVP NCLIV 058760 IADDN YWVG VG F AG F VEVHFAK TWVA TYSA CSUI_003845 LADES IDVQLSR Cyc_02015 EAH_00055290 MVDEN RLDIHLTF SFAA TWTA HTEE LLRLDVQLQR VGRIDISSSL RGIRKADFQL TVGPH IATTN ENH 00009810 LLH

b

SN3_01000185 AgsA_Salmonel

HSP20 Xylella

Extracellular parasites

RKTE

HF

ERRYGSFHR

1333543334

LNITG



Intracellular parasites



Supplementary Fig. 7 GRA45 alignment and its subcellular localization.

(a) Alignment of GRA45 from *Toxoplasma gondii* type I (TGGT1_316250), II (TGME49_316250) and III (TGVEG_316250); *Toxoplasma gondii* TGRUB_295390; *Hammondia hammondi* HHA_316250; *Neospora caninum* NCLIV_058760; *Cystoisospora suis* CSUI_003845; *Sarcocystis neurona* SN3_01000185; *Eimeria acervulina* EAH_00055290; *Eimeria necatrix* ENH_00009810; *Cyclospora cayetanensis* cyc_02015; HSP20 from *Xylella fastidiosa*; and AsgA from *Salmonella typhimurium*. Red boxes and blue boxes indicate predicted α -Crystallin domain (ACD) and predicted transthyretin-like fold, respectively. Predicted conserved secondary structures are indicated with red lines for helix and blue lines for strand. Green triangles and black triangles indicate the TEXEL motif and I/VxI/V motifs (<u>VKV</u> from amino acid 139 to 141, <u>VEV</u> from amino acid 162 to 164, <u>IDV</u> from amino acid 205 to 207, and <u>IDV</u> from amino acid 291 to 293), respectively. (b) Extracellular parasites (left panel) or intracellular parasites (right panel) expressing endogenously HA-tagged GRA45 were fixed, permeabilized, and subjected to immunofluorescent assays with the indicated antibodies. (scale bar = 2 µm for extracellular and 5 µm for intracellular parasites). The images are representative of results from 2 independent experiments.



Supplementary Fig. 8

(a) Insoluble GRA2 was determined by the percentage of volume intensity of high molecular weight (left) or monomer (right) from the pellet fraction in both pellet and supernatant fraction from Fig. 4a. Data are displayed as mean \pm SEM with independent experiments (n = 4) indicated by the same color dots. The significant difference was analyzed with two-tailed paired *t* test.

(b) Insoluble GRA7 was determined by the percentage of volume intensity of high molecular weight (left) or monomer (right) from the pellet fraction in both pellet and supernatant fraction from Fig. 4b. Data are displayed as mean \pm SEM with independent experiments (n = 4) indicated by the same color dots. (c) Extracellular parasites of WT or $\Delta gra7$ parasites were disrupted by freeze/thaw cycles followed by fractionation with high-speed centrifugation to separate the pellet (P) and supernatant (S). GRA7 were detected with anti-GRA7 antibodies. SAG1 was used as the parasite loading control. The image is result from 1 independent experiments.

(d) Extracellular WT parasites expressing endogenously 3xHA-tagged MYR1 or Δ*gra45* parasites in this background were disrupted by freeze/thaw cycles followed by incubating with PBS, 1% NP-40 or 1% Triton X-100 and fractionated by high-speed centrifugation to separate the pellet (P) and supernatant (S). The C-

terminal polypeptide of MYR1 was detected with anti-HA antibodies. SAG1 was used as the parasite

loading control. The image is result from 1 independent experiments.

(e) Insoluble MYR1-3xHA was determined by the percentage of volume intensity of pellet fraction in both pellet and supernatant fraction.



Supplementary Fig. 9 Quantification of GRA7, MAF1, GRA16 and GRA24 in Fig.5

(a) Extracellular $\Delta gra45$ or $\Delta gra45 + GRA45HA$ parasites were fixed with methanol and stained with the antibodies against GRA7. On the left, the representative images are identical to Fig.5b with longer exposure (Scale bar = 2 µm). Quantification of GRA7 intensity is presented in the right panel. Data are displayed as mean ± SEM with individual dots representing single parasites (n = 27 for $\Delta gra45$ parasites and n = 40 for $\Delta gra45 + GRA45HA$ parasites examined over 2 independent experiments). (b) HFFs were infected with $\Delta gra45$ or $\Delta gra45$ complemented with wild-type or indicated mutant version of *GRA45* for 24 h followed by fixing and staining with antibodies against MAF1. The images are representative of results from 2 independent experiments and were taken at identical exposure times for each channel (scale bar = 5 µm). The images are representative of results from 2 independent experiments.

(c) Localization of MAF1 from Fig.5d and (b) in at least 100 vacuoles was observed and the percentage of vacuoles with only PV lumen staining was quantified and presented in the table.

(**d** and **e**) Δ *gra45* or Δ *gra45* complemented with wild-type or indicated mutant version of *GRA45* were transiently transfected with GRA16-Ty (d) or GRA24-Ty (e) expressing plasmids and immediately used to infect HFFs and fixed at 24 h p.i. and subjected to the immunofluorescent assay with antibodies against the Ty epitope. The nuclear intensity of GRA16 (d) or GRA24 (e) was quantified in host cells containing a

single PV with 4 or more parasites. Data are displayed as mean \pm SEM with individual dots representing single host cell ($n \ge 40$ cells examined over 1 independent experiment).



Supplementary Fig. 10 All uncropped Western blot images in this study.