Rapid control of protein level in the apicomplexan *Toxoplasma gondii*

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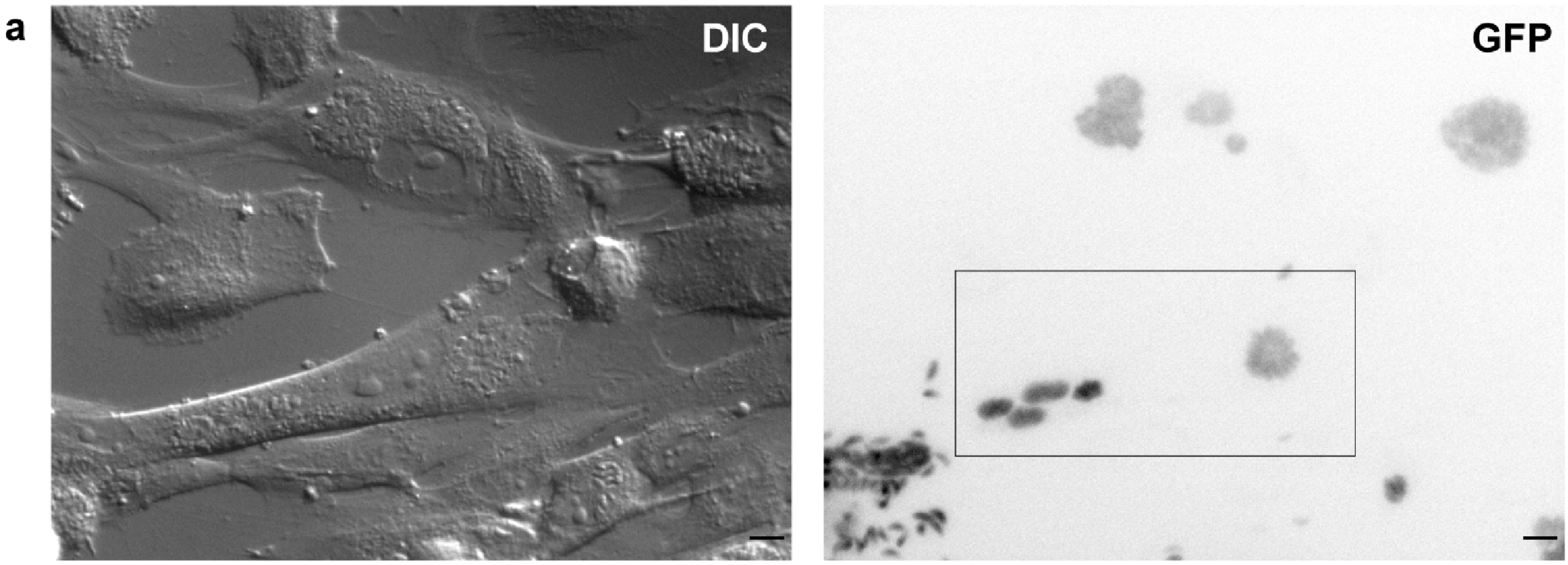
Supplementary figures and text:

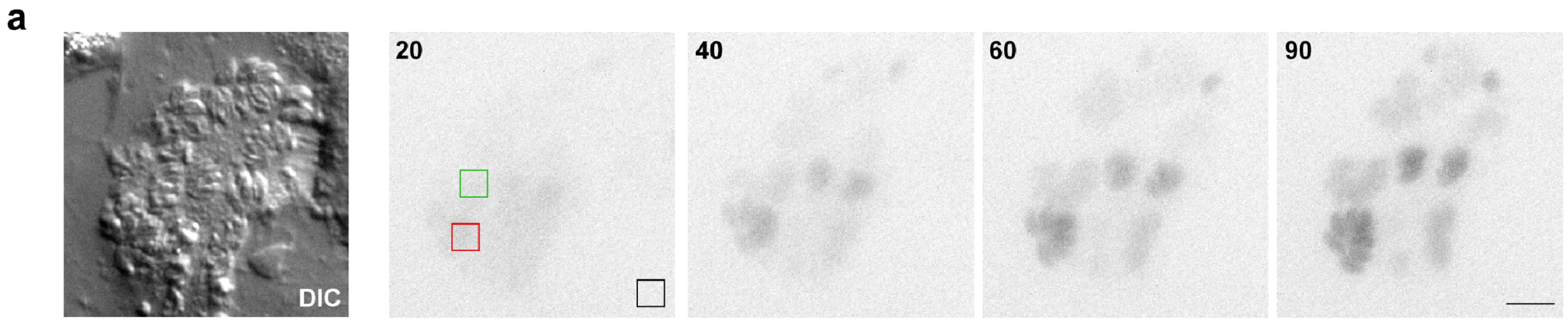
Note: Supplementary Movies 1 and 2 are available on the Nature Methods website.

Supplementary Figure 1. Quantitative Analysis of DD-YFP downregulation



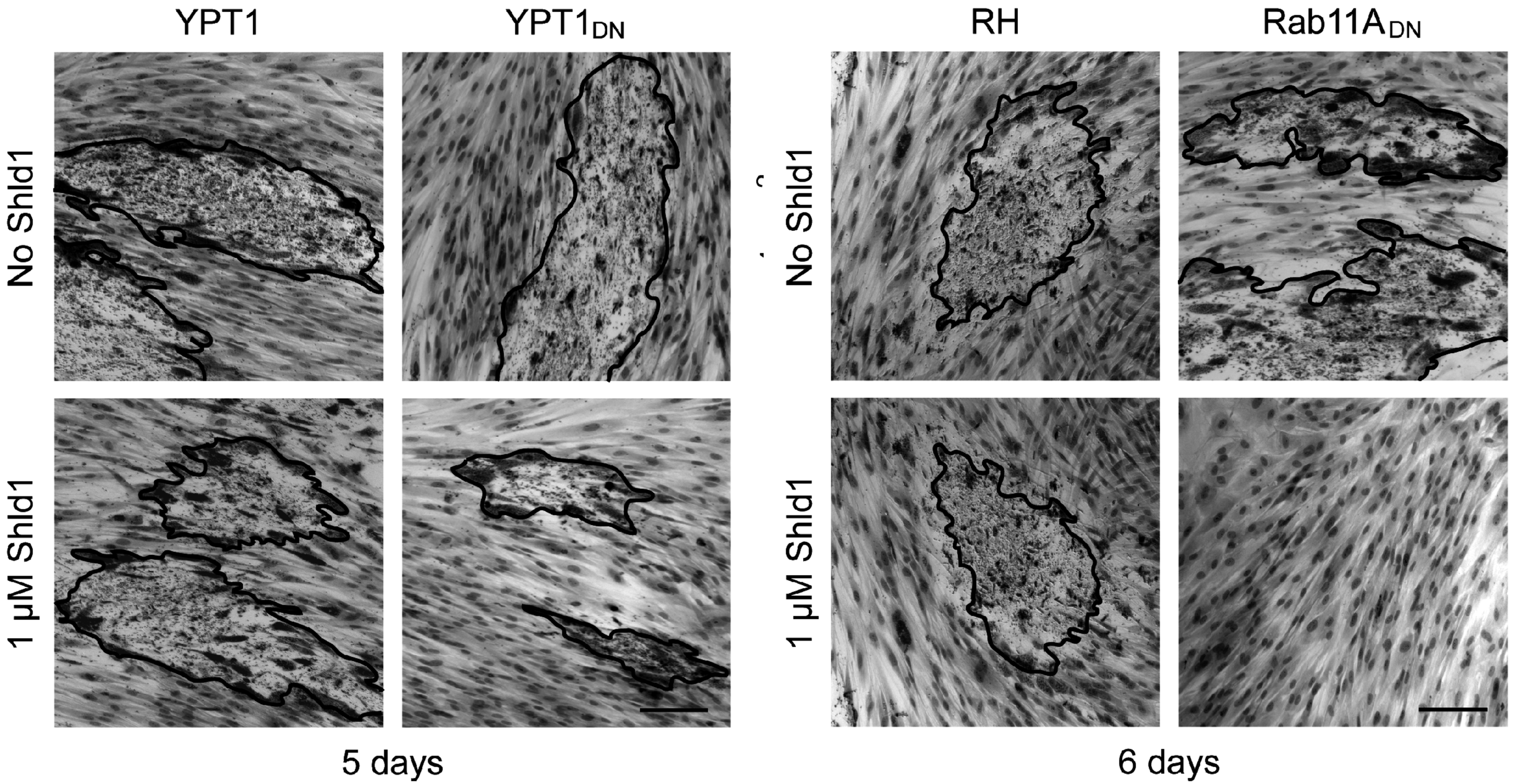
(a) DIC and inverted GFP image of HFF cells infected with DD-YFP parasites. (b) Images of the boxed area in (a) at different timepoints (indicated in minutes) during downregulation. After removal of Shld1 image were taken every 10 min during a total time of 7 hours. (c) Fluorescence intensities for the indicated parasites (coloured squares in b) were measured for the entire sequence and plotted for every timepoint (colours correspond to the squares). Black square and line correspond to background fluorescence. (d) Two additional examples for DD-YFP downregulation. The parasites were classified into two different populations. Red: parasites with a high level of fluorescence and green: parasites with a lower level of fluorescence at the beginning of the experiment. (e) The same measurement of fluorescence intensities was performed for 17 parasitophorous vacuoles from independent experiments. 13 parasitophorous vacuoles were classified as high expressers and merged into the red curve, 4 parasitophorous vacuoles were classified as low expressers and plotted in green (background was plotted in black). Error bars show standard deviation from the mean. Scale bars: 10µm.

Supplementary Figure 2. Quantitative Analysis of DD-YFP upregulation



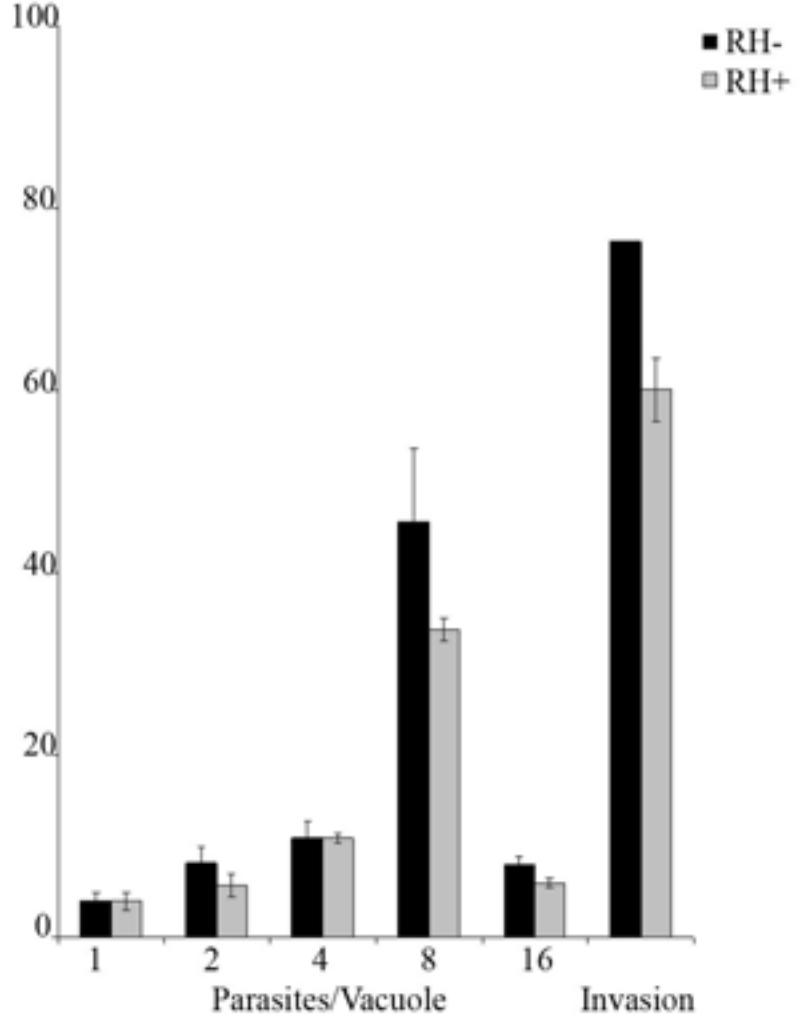
(a) left panel: DIC image of a HFF cell infected with DD-YFP expressing parasites. right panels: inverted fluorescence images of different timepoints during upregulation (time indicated in minutes). Acquisition started 20 min after addition of Shld1. Scale bar: 10 µm. (b) Fluorescence intensities for the indicated parasites (coloured squares in a) were measured for the entire sequence and plotted for every timepoint. Black square and line correspond to background fluorescence. (c) Similar analysis as described for the downregulation in suppl. Fig.1: The cells were classified according to their increase of fluorescence during the timecourse into 3 categories normalized according to the background and plotted together (green: 19 and orange: 2 vacuoles). Only one parasitophorous vacuole reached a plateau after about 70 minutes.

Supplementary Figure 3. Growth analysis of parasites strains DD-YPT1 and DD-YPT1DN or wildtype (RH) and DD-Rab11ADN.



Parasites were inoculated on HFF monolayers in presence and absence of Shld1 for 5 or 6 days before formation of plaques in the HFF-monolayer was compared. The plaque size was determined by defining the border of each plaque and calculation of the respective area as indicated by a black line. Scale bar: 50 µm.

Supplementary Figure 4. Addition of Shld1 has no effect on invasion or replication of wildtype parasites.



Quantification of invasion and replication of RHwt parasites (-: parasites not treated with Shld1; +: parasites treated with Shld1 prior to and post invasion). Total number of parasitophorous vacuoles and number of parasites per parasitophorous vacuole were determined. Mean values of 3 independent experiments +/- s.d. are shown.

Methods

Parasite cell lines and selections. T. gondii tachyzoites (RH *hxgprt*–) were grown in human foreskin fibroblasts (HFF) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine and 25 μg/ml gentamicin. To generate stable transformants, 5×10^7 freshly released RH*hxgprt*– parasites were transfected and selected in presence of mycophenolic acid and xanthine as previously described [1]. The selection based on pyrimethamine and chloramphenicole resistance were achieved as described previously [2,3].

Generation of constructs

To generate a construct expressing DD-YFP we amplified the DNA encoding the DD-domain using vector pBMN FKBP L106P-PpLuc [4] as template with oligonucleotides DD-s(BglII) 5′-CCAGATCTAAAATGGGAGTGCAGGTGGAA-ACCATC-3′ and DD-as(AvrII) 5′-GGCCTAGGTTCCGGTTTTAGAAGCTCCACATCG-3′ and inserted it into the vector tubYFP-YFP/sagCAT [5] via AvrII/BglII. Subsequently oligonucleotides encoding a myc-epitope tag were inserted into the single AvrII site (myc-sense 5′-CTAGCATGCAGGAGCAGAAGCTCATCTCCGAGGAGGACC-TGGCCATGGCCATGCATC-3′ and myc-as 5′-CTAGGATGCATGGCCATGGCCA-GGTCCTCCTCGGAGATGAGCTTCTGCTCCTGCATG-3′), resulting in the vector tubDDmycYFP-CAT.

To generate a construct expressing GFP-DD, DNA encoding DD-domains was amplified using oligonucleotides DD-s(PstI) 5′-CCCTGCAGGAGTGCAGGTGGAAACCATC-3′ and DD-as(PacI) 5′-GGTTAATTAATTATTCCGGTTTTAGAAGCTCCACATCG-3′ and introduced into the PstI/PacI sites of vector p5RT70mycGFP/HX [6], resulting in the vector p5RT70mycGFP-DD/HX.

In order to express DD-mycMyoA vector tubDDmycYFP-CAT was digested with EcoRI/NsiI, the fragment corresponding to DD-myc was isolated and inserted into the respective sites of p5RT70mycMyoA [6], resulting in construct p5RT70DDmycMyoA.

For expression of dominant negative DD-Rab11A, cDNA encoding Rab11A was generated and cloned into pGEM-vector (Promega, Madison, USA) by RT-PCR using Oligonucleotides Rab11-s(NsiI) 5′- CCATGCATGCGGCTAAA-GATGAATACTACG-3′ and Rab11-as(PacI) 5′- CCTTAATTAACAGGCGGAA-CAGCAGCCACGTC-3′. Subsequently a single point mutation was generated (N126>I) by overlapping PCR-s using oligo pairs Rab11-s(NsiI)/Rab11-as(mut) 5′- GGATCCGCATGGTCGCGAAGCTC-3′ and Rab11-

as(PacI)/Rab11-s(mut) 5´-GGATCCCAACATCGTCATTCTGCTCGTGGGAATCAAAAG-CG-3´. The resulting PCR-fragments were relegated via BamHI and inserted into NsiI/PacI of p5RT70DDmycMyoA, resulting in vector p5RT70DDmycRab11m. For expression of heterologoues YPT1 and dominant negative YPT1(N121>I) from *Candida albicans* PCR was performed on pYPT1 and pN121I [7] with oligonucleotides YPT1-s(NsiI) 5´-GCCATGCATATGAATAACGAATACG-3´and YPT1-as(PacI) 5´- GCCTTAATTAACA-ACAGGAATTCGATTGG-3´ respectively. The resulting fragments were inserted into NsiI/PacI of p5RT70DDmycMyoA, resulting in construct p5RT70DDmyc-YPT1 and p5RT70DDmyc-YPT1m. For stable selection *HXGPRT* was excised from p5RT70mycGFP/HX[6] and placed in between BamHI and SacI of the respective vectors.

Generation of parasite strains

Parasites expressing DDYFP were generated by stable transfection with tubDDmycYFP-CAT employing chloramphenicole selection. Parasites expressing GFP-DD , DDmycRab11A$_{DN}$, DDmycYPT1 and DDmycYPT1$_{DN}$ were transfected with the respective vector and selected for HXGPRT-expression. Parasites expressing DDmycMyoA were generated by co-transfection of the respective expression vector with pDHFR-Tsc [8] and selected for pyrimethamine resistance.

Immunoblot assays

Intra- or freshly lysed extracellular parasites were incubated in culture media in the absence or presence of 1 µM Shld-1 and incubated as indicated. Subsequently parasites were harvested and washed once in ice cold PBS. SDS – PAGE and Western Blot analysis were performed as described previously [6], using 6 – 12% polyacrylamide gels under reducing condition with 100 mM DTT. Per experiment an equal number of parasites were loaded. For detection polyclonal FKBP12 (ABCAM, Cambridge, UK), monoclonal c-myc (9E10, Sigma-Aldrich, USA) or polyclonal anti-TgMyoA [9] were used. As internal control monoclonal anti-MIC2 [10] or polyclonal anti-Tub1 [11].

Immunofluorescence Assays

All manipulations were carried out at room temperature, if not mentioned otherwise. Tachyzoite-infected HFF cells on glass coverslips were fixed with 4% formaldehyde in PBS for 20 minutes, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes and blocked in 3% bovine serum albumin in PBS for 20 minutes. The cells were then stained

with primary antibodies (see above), as indicated in the text, for one hour followed by Alexa 594 goat anti-rabbit or Alexa 488-conjugated goat anti-mouse antibodies (Molecular Probes, Inc.). Images were taken using a Zeiss microscope (Axiovert 200M) with a 63x objective and processed using ImageJ 1.34r software.

Growth assays

The plaque assay was performed as described before [12]. Monolayers of human foreskin fibroblasts (HFF), grown in 6 well plates, were infected with 50 to 100 tachyzoites per well. After one weak of incubation at normal growth conditions (37°C, 5% CO_2), cells were fixed 10 minutes with –20°C methanol 100%, dyed with Giemsa stain for 10 minutes and washed once with PBS. Images were taken using a Zeiss microscope (Axiovert 200M) with a 10x objective and plaque size was determined using Axiovision software (Zeiss). For each strain the sizes of at least 20 plaques was measured and mean values were calculated according to standard procedures [13].

Invasion and Replication assay

Assays were performed as previously described[14]. Briefly, 5×10^6 freshly egressed parasites were incubated for 20 minutes in presence or absence of Shld-1, before inoculation on host cells. Parasites were allowed to invade for 3 hours in presence and absence of Shld-1 and subsequently three washing steps to remove extracellular parasites were performed. Cells were then further incubated for 18 hours in presence and absence of Shld-1 before fixation. The number of vacuoles representing successful invasion events was determined in 20 fields of view and the number of parasites per vacuole was determined. The number of vacuoles represents a percentage of 100% (which reflects successful invasion) in the absence of Shld-1. Mean values of three independent experiments +/- S.D. have been determined.

Live cell imaging

HFF cells were grown on 35mm glass bottom culture dishes (MatTek, Ashland, MA, USA) in complete Dulbecco's Modified Eagle's Medium (DMEM) (10% FCS). The cells were infected with DD-YFP parasites and grown overnight in the absence or presence of 1µM Shld-1. Before imaging, cells were washed and incubated in complete DMEM without phenol red (with 10% FCS). Culture dishes were then transferred to the stage of a Zeiss an inverted microscope (Axiovert 200M "Cell observer") with a temperature and CO_2 controlled incubator at 37°C and 5% CO_2. For upregulation experiments, 1µM Shld-1 was added directly

into the imaging medium on the microscope stage. For downregulation experiments cells were simply transferred into preheated medium without Shld-1. Imaging was performed with a 40x objective (LD Plan-Neofluar, N.A. 0.6, Zeiss) and images were captured with an AxioCam HRm (Zeiss). Image acquisition was performed with Axiovision (Zeiss). Fluorescence intensities of parasitophorous vacuoles were measured over a 50/50 pixel square for all acquisitions (up- and down regulation) using a modified version of the histogram plugin from ImageJ.

FACS analysis

Parasites grown in presence of different Shld-1 concentrations were harvested and 100.000 data points were collected for each sample in flow cytometry using FACSEXCALIBUR (Becton Dickinson Immunocytometry Systems, San José, CA, USA). Data were analysed using CellQuest Software 4.0.2 (Becton. and Dickinson, UK) and WinMDI 2.9 (http://facs.scripps.edu/)

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