A Genome-wide CRISPR-Cas9 Screen Identifies Host Factors Essential for Optimal *Plasmodium* Liver Stage Development.

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Summary

Prior to initiating symptomatic malaria, *Plasmodium* parasites infect and develop within hepatocytes. We performed a forward genetic, genome-wide CRISPR-Cas9 screen to identify host regulators of *Plasmodium* liver infection. Single guide RNAs targeting genes involved in vesicle trafficking, cytoskeleton organization and lipid biogenesis altered *Plasmodium* liver development. We observed a redistribution of Golgi-derived vesicles and fragmented Golgi stacks with the parasitophorous vacuolar membrane (PVM). The host microtubule network and non-centrosomal microtubule organizing centers (ncMTOC) also re-localized following infection, closely associating with the parasite. Knocking out the centrosomal MTOC protein CENPJ exasperated the re-localization of MTOCs to the parasite and increased infection, suggesting that the parasite relies on ncMTOC assembly. Thus, we have uncovered a mechanism by which parasites sequester host material for survival and development. Our data provide a wealth of yet untested hypotheses about the elusive biology of the liver stage parasite and serves as a foundation for future investigation.

Introduction

Malaria is transmitted to humans by the injection of *Plasmodium* sporozoites into the skin during the blood meal of an infectious female *Anopheles* mosquito. Sporozoites exit the skin by traversing blood vessels to enter the circulation and are then carried to the liver. Sporozoites leave the circulation by traversing the sinusoidal cell layer and infecting hepatocytes and forming liver stages (LS) (Mota et al., 2001; Shortt and Garnham, 1948; Vanderberg, 1981). LS parasites reside in a membrane-bound compartment in the hepatocyte termed the parasitophorous vacuole (PV), where they differentiate into

exoerythrocytic merozoites. The PV membrane subsequently breaks down, and merozoites reenter the blood and infect erythrocytes (Sturm et al., 2006).

Hepatocyte infection is obligate for parasite life cycle progression, and thus is an important target for antimalarial intervention. Elimination of the parasite during this stage would block both disease symptoms and transmission. Several studies have shown that *Plasmodium* relies on multiple surface factors including CD81 (cluster of differentiation 81) (Silvie et al., 2003), SRB1 (scavenger receptor class B type 1) (Rodrigues et al., 2008) and EphA2 (ephrin type-A receptor 2) (Kaushansky et al., 2015) for hepatocyte entry. Host factors also effectively control *Plasmodium* hepatocyte development after entry, including aquaporin 3 (Posfai et al., 2018), phosphatidylcholine (Itoe et al., 2014), apolipoprotein H (Sa et al., 2017), p53 (Kaushansky et al., 2013), XIAP (X-linked inhibitor of apoptosis protein) (Ebert et al., 2020), LC3 (a ubiquitin-like protein derived from posttranslational modifications of microtubule-associated proteins 1A/1B light chain 3A) (Real et al., 2018) and heme oxygenase (Epiphanio et al., 2008). Yet, a comprehensive view of the molecular details that regulate the establishment of LS infection, and how these regulatory factors modulate one another during infection, remains elusive.

Multiple forward genetic screens have informed our understanding of host regulatory factors for LS malaria. A kinome-wide screen identified five kinases that play a major role in *P. berghei* infection (Prudencio et al., 2008). More recently, we used an orthologous approach, kinase regression, and confirmed that many of the originally described kinases play a role in a related *P. yoelii* system. We also showed many additional kinases regulate *Plasmodium* LS infection (Arang et al., 2017). Separately, an RNAi screen against 53 targets identified SRB1 as an invasion factor (Rodrigues et al.,

2008), and a subsequent screen against 6,951 druggable targets identified COPB2 (coatomer subunit beta) and the ADP-ribosylation factor-binding protein GGA1 (Golgi-associated, gamma adaptin ear containing, ARF binding protein 1) as development factors (Raphemot et al., 2019) for *Plasmodium* LS infection. While these screens have provided valuable insights into parasite-host interactions, the range of targeted genes and overlap in the identified hits has been limited, suggesting that a complete complement of factors required for *Plasmodium* entry and development remains to be discovered.

The recent development of genome-wide CRISPR-Cas9 technology enables generation of complete loss-of-function alleles in a variety of cell types, enabling functional genetic analyses in higher eukaryotes (Sanjana et al., 2014; Shalem et al., 2014). Here, we report the first genome wide CRISPR-Cas9 screen that aims to identify novel host factors that regulate *Plasmodium* infection. Importantly, we identify pathways that overlap with previous screens, in addition to uncovering novel host regulators of *P. yoelii* LS infection.

Results

CRISPR-Cas9 screen to identify host regulators of infection

To identify host genes critical for *Plasmodium* LS development, we used the GeCKOv2 sgRNA pool to generate a whole-genome knockout library in HepG2-CD81 cells (Sanjana et al., 2014; Shalem et al., 2014). HepG2-CD81 cells were transduced with lentivirus containing the pooled GeCKOv2 sgRNA library of 114,123 sgRNAs targeting 19,031 protein-coding genes (~6 sgRNAs/gene) and selected in puromycin for 5-7 days. To evaluate sgRNA diversity in the HepG2-CD81-GeCKOv2 library, we PCR-amplified the

integrated sgRNA cassettes from genomic DNA extracted from transduced cells and subjected the amplified library to Illumina sequencing. At the gene level, sgRNAs targeting all but 15 of 19,031 (99.92%) protein-coding genes were observed. Twelve to fourteen days after post-transduction, we infected forty million puromycin-resistant cells with green fluorescent protein (GFP) expressing-*Plasmodium yoelii* at a multiplicity of infection (MOI) of 0.3. After 24 h of infection, cells were sorted into infected and bystander cell populations by GFP signal intensity with fluorescence-activated cell sorting (FACS) (Fig. 1A). Separately, a parallel culture of uninfected cells was also maintained to normalize the sgRNA frequency distributions. We obtained four independent biological replicates with library generation and sequencing occurring in parallel. Genes with significantly enriched sgRNAs were identified for both the bystander and infected populations, when compared to uninfected cells.

Cells that harbor genetic alterations restricting *P. yoelii* development (i.e., sgRNAs that target host genes important for infection) were expected to be enriched in the uninfected bin; we termed this group ‘putative positive regulators of infection’. We categorized sgRNAs enriched in the infected cells as ‘putative negative regulators of infection’. In this initial screen, we identified 242 sgRNAs that were statistically enriched in infected or bystander groups after accounting for multiple hypotheses. There were 67 sgRNAs significantly enriched in the infected cells compared to uninfected cells and 175 genes were significantly enriched in the bystander bin relative to uninfected bin. We reasoned that biological pathways with multiple putative regulators were more likely to be bona fide regulators of infection. We used gene ontology (GO) pathway analysis to identify significantly enriched biological processes (Fig. 1B). Genes present in both

statistically enriched pathways were selected for further validation. After this stringent down-selection step, we were left with eight putative negative regulators of infection and seven putative positive regulators of infection for further study (Fig, 1C, D).

Identifying host factors that regulate *Plasmodium* LS invasion and development

To distinguish between genes impacting *Plasmodium* LS invasion versus those impacting development, we individually disrupted each of the 15 putative regulators with three sgRNAs per gene using CRISPR-Cas9 gene editing of HepG2-CD81 cells. In this system, a fluorescent reporter, GFP, is expressed only upon guide integration, enabling us to exclude any cells that did not take up and integrate the sgRNA (Fig. S1A). Knockout efficiency of CENPJ was also confirmed using western blot (Fig. S1B and S1C). We infected each knockout line with *P. yoelii* sporozoites for 90 mins and assessed hepatocyte entry by flow cytometry. Among the selected 15 hits, only LRP4 (low-density lipoprotein receptor-related protein 4) exhibited significantly reduced entry of sporozoites 90 min after infection (Fig. 2A). This is consistent with the previous finding that CSP interacts with LRP and HSPG to facilitates host cell invasion of *Plasmodium* (Shakibaei and Frevert, 1996).

As an orthogonal approach, we modulated HepG2-CD81 cells with small molecule inhibitors targeting positive regulators identified in the screen (Fig. 2B)(Supplementary Table 1). IC50 values for each small molecule inhibitor were obtained in HepG2-CD81 cells using Live/ Dead staining (Fig. S1D). We included eltanexer, an inhibitor of exportin-1 (XPO1), a pututive negative regulator of infection (Than et al., 2020). To test the role of LRP4 in sporozoite entry of hepatocytes, we pretreated HepG2-CD81 cells with HPA-12, a ceramide transport inhibitor (Berkes et al., 2016) and an LDL-R blocking peptide which

blocks LRP4, significantly inhibited sporozoite entry. Thus both genetic and peptide-mediated intervention of LRP4 inhibits sporozoite entry of hepatocytes (Fig. 2A and 2B).

We next performed an imaging-based secondary screen with the selected 15 putative regulators to assess the role of these hits on the longer-term LS development. Individual CRISPR-Cas9 knockout lines were infected with *P. yoelii* sporozoites and observed for 48 hours post infection (hpi). Several of the knockout lines exhibited substantially altered LS burden (Fig. 2C). The number of LS parasites was significantly increased in CENPJ (centromere protein J) and KDELC1 (Lys-Asp-Glu-Leu containing 1) disrupted lines, illustrating that each of these factors is indeed a negative regulator of infection. In contrast, knockout of CISD1 (CDGSH iron sulfur domain 1), COL4A3BP (collagen type IV alpha-3-binding protein), CERT1 (ceramide transporter protein 1), IREB (iron-responsive element-binding protein) and LRP4 significantly reduced the number of LS parasites 48 hpi (Fig. 2C). We next tested whether LS infection could be perturbed by targeting these factors with pharmacological inhibitors. Consistent with the genetic experiments, the battery of small molecule inhibitors (Supplementary table 1) that have been shown to target CISD1, COL4A3BP (CERT1), IREB, and LRP4, significantly reduced the number of LS parasites observed after 48 h of infection (Fig. 2D), further supporting the notion that these factors are positive regulators of LS infection.

We next asked if any of the screen hits altered the growth of LS parasites. Interestingly, knock out of VPS51 (Ang2) did not significantly alter parasite load but instead, the size of the parasite was significantly smaller suggesting it may regulate parasite growth. HepG2-CD81 cells expressing sgRNAs directed against COL4A3BP and LRP4 exhibited a significant reduction in the size of the parasite (Fig. 2E). Knockout of

other putative regulators did not result in altered parasite size (Fig. 2E). Interestingly, while our screen was only set up to identify factors that altered infection rate, not LS growth, it is possible that some slow-growing parasites may have not reached the threshold of GFP levels to be included in the “infected” gate. Taken together, these studies identified several host factors influencing parasite entry, growth and development (Fig. 2F) and illustrates the utility of genome-scale functional screening for the discovery of host factors that regulate *Plasmodium* LS infection.

Host Golgi and Golgi-derived vesicles interacts with *Plasmodium*

Interestingly CERT1, a positive regulator, and VPS51, a negative regulator, were both involved in trafficking to and from the Golgi. Specifically, CERT1 regulates ER-to-Golgi and Cis Golgi-to-trans Golgi network (TGN) traffic (Funakoshi et al., 2000). VPS51 (Ang2) regulates late endosome-to-TGN and Golgi-to-ER traffic (Perez-Victoria et al., 2010). Given this, we hypothesized that targeting vesicle trafficking to and from Golgi impacts parasite survival. Moreover, GO term enrichment analysis (Fig. 1B) suggests cytoskeleton organization, vesicular trafficking and Golgi and ER stress significantly impact LS infection. Since increases in Golgi-stress and vesicular trafficking are associated processes (Sasaki and Yoshida, 2015), we chose to visualize the Golgi and Golgi-derived vesicles in infected cells. We infected HepG2-CD81 cells with *P. yoelii* sporozoites and allowed infection to proceed for 24 or 36 h. Cells were stained with anti-UIS4 (upregulated in infectious sporozoites gene 4) and anti-FTCD (formiminotransferase-cyclodeaminase) antibodies to visualize the parasite parasitophorous vacuole membrane and host Golgi-derived vesicles, respectively. We observed an enrichment of Golgi-derived vesicles near the parasite (Fig. 3A), consistent

with other reports (De Niz et al., 2020; Raphemot et al., 2019). To assess Golgi morphology during infection, we infected HepG2-CD81 cells with *P. yoelii* sporozoites and stained cells with antibodies against the Golgi peripheral cytoplasmic membrane protein, GM130 (Golgi membrane protein of 130 kDa; golgin subfamily A member 2). As a control for Golgi fragmentation, we used brefeldin A, which reversibly disrupts and fragments the Golgi, blocking assembly and transport of secretory vesicles (Sciaky et al., 1997). We observed fragmented Golgi stacks in infected cells 24 hpi, which resolved by 36 hpi (Fig. 3B). We also observed UIS4-positive membrane positioned near the Golgi stacks in nearly three quarters of the infected cells at 24 hpi (white arrowheads, Fig. 3B). This observation suggests that the PVM or the tubulo-vesicular network is positioned near the Golgi stacks and/or Golgi-derived vesicles.

Plasmodium **hijacks the host microtubule network**

Several studies have reported an association between LS parasites and host derived vesicles, including late endosomes (Lopes da Silva et al., 2012), autophagosomes (Real et al., 2018) and lysosomes (Niklaus et al., 2019; Risco-Castillo et al., 2015; Vijayan et al., 2019). Microtubules (MTs) support the spatial positioning and vesicular trafficking of these and other organelles (Vale, 2003). Genes with roles in MT cytoskeletal organization were associated with a series of GO terms enriched in our screen (Fig.1B). Thus, we next asked if the localization of host vesicles, including Golgi-derived vesicles, at the *Plasmodium* PVM, was regulated by the host MT network. Interestingly, the Golgi acts as a MT organizing center (MTOC) by recruiting γ-tubulin with a subset of MT originating at the Golgi in mammalian cells, and across many systems (Sanders and Kaverina, 2015). To visualize changes in the MT network initiated during infection, we transfected

HepG2-CD81 cells with CellLight™ RFP-α-Tubulin BacMam 2.0 and allowed the infection to proceed for 24 h (Fig.4A). Strikingly, we observed that the host MT network redistributes to, the LS parasite, appearing to wrap around the PVM (Fig.4A). In contrast, MTs in uninfected cells form a canonical network around the nucleus, radiating toward the cell periphery (Fig.4A).

Acetylated MT are the stabilized form of MTs that support kinesin-mediated trafficking of vesicles (Reed et al., 2006). We next asked if parasite associated MTs are actively trafficking by assessing the acetylation of MTs. We infected HepG2-CD81 cells with *P. yoelii* sporozoites for 24 hours, and then visualized acetylated alpha-tubulin and *Py*UIS4 by immunostaining (Fig.4A). MTs that decorate the parasite periphery were highly acetylated. In contrast, in uninfected cells, acetylated MT were scattered throughout the cell. These results are consistent with a model where the MT network drives elevated levels of vesicular traffic to the parasite periphery in infected cells.

Non-centrosomal microtubule organizing centers sequester at parasite periphery.

To understand MT redistribution in infected cells, we visualized MT organizing centers (MTOCs) (Zheng et al., 1995) and assessed their role in regulating MT nucleation and growth during infection. MT arrays originate from MTOCs that are either organized canonically at the centrosome or non-canonically at Golgi (Sanders and Kaverina, 2015; Zhu and Kaverina, 2013). Centrosome-regulated MTs are strictly radial while non-centrosomal organized MTs display asymmetrical organization (Zhu and Kaverina, 2013). Given the non-canonical organization of MTs around the PVM and the redistribution and relocalization of Golgi-derived vesicles with the PVM, we hypothesized that MTs organized around the parasite were non-centrosomal. To test this, we evaluated the

localization of calmodulin-regulated spectrin-associated protein 2 (CAMSAP2), a minus-end binding protein that stabilizes non-centrosomal MTs (Yau et al., 2014). In uninfected CellLight™ GFP-α-Tubulin BacMam 2.0 transfected HepG2-CD81 cells, CAMSAP2 found distributed evenly throughout the cells. Strikingly, we observed enrichment of CAMSAP2 on the MTs around the PVM suggesting that parasite localized MTs are non-centrosomal, consistent with the hypothesis that they are stabilized at the parasite periphery (Fig.4B).

We next asked how MTs were organized in infected cells by assessing the localization of γ-tubulin with γ-TuRC (γ-tubulin ring complex). γ-TuRC is a core functional unit of MTOCs and functions as a MT nucleator (Wiese and Zheng, 2000), usually localized to the centrosome. In uninfected cells, γ-tubulin was localized primarily near nuclear periphery (88%). In infected cells, a majority of γ-tubulin foci (~60%) were found in the cytoplasm associated with the PVM. During cell maturation, γ-TuRC gradually shifts localization from the centrosome to the cytoplasm and nucleates MTs . Together, the association of the parasite with the Golgi, re-localization of γ-TuRC to the cytoplasm, and the observed localization of CAMSAP2 to the PVM, supports the hypothesis that MT nucleation around the parasite is of ncMTOC origin.

We next evaluated the role of CENPJ, which is involved in establishing centrosomal MTOCs (Hung et al., 2000), in assembling ncMTOCs in infected cells. We generated CENPJ disrupted lines in HepG2-CD81 cells (Fig. S1C and D). In CENPJ disrupted cells, we observed an increase in cytoplasmic localization of γ-tubulin (~80%). Infection in CENPJ knockout cells resulted in an increase in γ-tubulin localization (~92%) to the PVM compared to infected control cells (~60%) (Fig. 4B) and an increase in LS

infection (Fig.2C). This is consistent with a model where the parasite interacts with host Golgi, and Golgi-associated ncMTOCs to traffic host vesicles to the PVM and promote LS development.

Integrating multiple forward genetic screens provides additional testable hypotheses

We report the first global screen for host factors that regulate *Plasmodium* LS infection. Yet, like previous screens, our screen includes false negatives as sgRNAs are lost during the generation of the library and/or not all sgRNAs result in the disruption of the functional protein. To generate a more comprehensive picture, we systematically compared our screen, which interrogated host regulators of *P. yoelii* infection, with earlier forward genetic screens (Prudencio et al., 2008; Raphemot et al., 2019; Rodrigues et al., 2008) that identified regulators of the closely related parasite, *P. berghei* (Fig. 5A, Supplementary file 1). For the purpose of analysis, we pooled results from the screens by Rodrigues et al., 2008 and Prudencio et al., 2008, as these two screens used the same methodology but had no overlapping factors evaluated. A meta ranking of the 3 screens was performed by sorting each screen separately by z score, calculating each gene's rank percentile location after sorting, and then averaging the gene rank percentile locations across the 3 screens, with no penalty for a gene being missing in a screen. This meta ranking was lastly sorted by average rank percentile location and augmented with the average z score from all screens where the function of the gene was evaluated (Supplementary file 1). Positively and negatively represented genes were sorted separately, then combined afterwards for pathway analysis. At gene level, using the z score cutoff of 2 and 1.5, our screen shared only few hits each of the other screens (Fig.

5B and C). When we loosened the stringency of the cutoff to a Z-score of 1, there were several genes overlapping between the three screens, although false positive rates could be higher at this cut-off.

We reasoned that hits from overlapping pathways and biological processes might be present in all screens, despite little specific gene overlap. To gain insights into the biological significance of the hits from all screening efforts, we employed ClueGO to determine gene ontology (GO) (Bindea et al., 2009). Despite little or no overlap in specific gene hits, we observe significant enrichment in biological processes from the genes represented in at least two of three screens at z-score of 1.5 and 1. Specifically, we identified 19 high confident biological processes that are significantly enriched using a z-score cutoff of 1.5. This includes biological processes that have been previously described, such as scavenger receptor activity and cholesterol biosynthesis reported (Itoe et al., 2014; Labaied et al., 2011; Petersen et al., 2017; Rodrigues et al., 2008) as well as anterograde cargo transport that we have interrogated in more detail in this study (Fig. 5D and E). Taken together, this combined resource provides a wealth of hypotheses for further investigation.

Discussion

For decades, dogma suggested that the elimination of anything short of 100% of LS parasites would result in little to no benefit in the effort towards malaria eradication. New evidence suggests this is not the case. Mathematical models suggest elimination of even a portion of hypnozoites could dramatically reduce *P. vivax* prevalence (White et al., 2018). Recently, it was demonstrated that targeting host aquaporin 3 leads to the elimination of *P. vivax* hypnozoites from field isolates (Posfai et al., 2020), suggesting that

host targeted interventions may provide an opportunity to tackle even the Achilles heel of malaria control efforts. Another host factor known to be critical for stages infection, the tumor suppressor p53 (Kain et al., 2020; Kaushansky et al., 2013), has been associated with lower severity of infection in Malian children (Tran et al., 2019). Additionally, it was recently demonstrated that host targeted interventions can induce at least partial immunity to subsequent challenge (Ebert et al., 2020). This opens the door to the possibility of the use of host targeted drugs in casual prophylaxis strategies, provided the drugs have suitable toxicity profiles (reviewed in (Glennon et al., 2018)). Because not all host targets are suitable drug targets, a broad and comprehensive picture of factors that regulate LS malaria infection is urgently needed.

Previous forward-genetic screens have partially provided that picture and identified host factors involved in *Plasmodium* infection (Prudencio et al., 2008; Raphemot et al., 2019; Rodrigues et al., 2008). However, these screens have exhibited very little overlap in identified factors (Fig. 5), presumably in part because each screen prioritized identifying a small, but bona fide list of “hits,” and suffered a high false negative rate as a result. This has led to many key insights into the interactions between the malaria parasite and its host hepatocyte but has fallen short of providing a systematic view of the fundamental biological properties that regulate the development and survival of the LS parasite. Like the earlier screens, the CRISPR-Cas9 screen we report here does not exhibit substantial overlap with previous screens when individual gene hits are evaluated, suggesting that additional analysis is still needed in order to comprehensively assess factors that regulate infection. Yet, when we evaluate whether hits from our screen are present in similar pathways to those observed in other screens, the overlap is substantial (Fig. 5D, E). This

suggests that while we may have, as a field, identified many central regulatory biological functions that control LS development, we have yet to saturate our understanding of the molecular players that mediate these biological necessities. Together, the CRISPR-Cas9 screen we present here, along with the previously reported siRNA screens, represent a key resource for the field moving forward, and we anticipate that merging findings from these experiments (Supplemental File 1) will provide many additional hypotheses to probe. One limitation of this work is that, since it is likely that at least a subset of canonical signaling pathways are rewired in the course of infection (Glennon et al., 2019), pathway analysis, which is based primarily on canonical signaling networks, is unlikely to comprehensively describe the an entirely accurate topology of the signaling relationships that mediate the complex host-parasite interface. Developing tools to reconstruct signaling relationships, within the context of malaria infection, is a critical area for future investigation.

Multiple screening efforts and other reports (Lopes da Silva et al., 2012; Niklaus et al., 2019; Petersen et al., 2017; Prudencio et al., 2008; Raphemot et al., 2019; Real et al., 2018; Rodrigues et al., 2008) have pointed to the critical role of vesicular transport, more specifically retrograde trafficking (Raphemot et al., 2019), throughout *Plasmodium* LS infection. Here, we observe an association of Golgi and post-Golgi anterograde vesicles with the parasite, suggesting the parasite sequesters both anterograde and retrograde trafficking of host derived vesicles. Our data are consistent with the model that these vesicles are trafficked via MTs to the parasite periphery as a result of ncMTOCs localized to the parasite periphery, likely assembled by the closely associated Golgi. This MTOC localization and subsequent trafficking provides a functional benefit to the parasite,

as knockdown of CENPJ, which inhibits centrosomal MTOC formation and further exacerbates the localization of ncMTOCs to the parasite periphery increases LS parasite infection. Interestingly, several reports previously identified the fusion of host derived vesicles such as late endosomes (Lopes da Silva et al., 2012), lysosomes (Niklaus et al., 2019), autophagosomes (Real et al., 2018) and retrograde vesicles from Golgi with the parasite (Raphemot et al., 2019) to enable development. Our data suggest that this fusion may be facilitated by MTOC localization to the parasite PVM and the resultant vesicular trafficking to the parasite periphery. Similar reorganization of the host MT network to facilitate the hijacking of host vesicles has been reported in *Toxoplasma gondii* (Coppens et al., 2006), suggesting that this may be a conserved mechanism by which Apicomplexan parasites exploit for nutrient uptake and survival.

Materials and Methods

Cell lines, plasmids and antibodies

HepG2-CD81 cells (Silvie et al., 2006) were maintained in DMEM-Complete Medium (Dulbecco's modified eagle medium (Cellgro, Manassas, VA), supplemented with 10% v/v FBS (Sigma-Aldrich, St. Louis, MO), 10000 IU/ml penicillin, 100 mg/ml streptomycin (Cellgro), 2.5 mg/ml fungizone (HyClone/Thermo Fisher, Waltham, MA) and 4 mM L-Glutamine (Cellgro). Cells were split 2-3 times weekly. All the plasmids for Genome-wide CRISPR library generation (GeCKOv2) and individual gene knockouts were procured from Addgene (MA, USA). CellLight Tubulin-RFP / GFP, BacMam 2.0 ready transfection mix were purchased from ThermoScientific (Missouri, USA). The following antibodies were used throughout this study: From Cell Signaling Technologies: acetylated alpha

tubulin (5335S); ThermoScientific: gamma tubulin (PA1-28042) and FTCD (PA5-83166 and Novus biologicals: GM130 (NBP2-53420) LSBio: UIS4 (LS-C204260-400).

Mosquito rearing and sporozoite production

For *P. yoelii* sporozoite production, female 6–8-week-old Swiss Webster mice (Harlan, Indianapolis, IN) were injected with blood stage *P. yoelii* (17XNL) parasites to begin the growth cycle. Animal handling was conducted according to the Institutional Animal Care and Use Committee-approved protocols. Briefly, *Anopheles stephensi* mosquitoes were allowed to feed on infected mice after gametocyte exflagellation was observed. Salivary gland sporozoites were isolated using a standard protocol at day 14 or 15 post-blood meal. The sporozoites were activated with 20% v/v FBS and pelleted by centrifugation at 1,000 × *g* to salivary gland detritus. Sporozoites were further enriched by a second centrifugation at 15,000 × *g* for 4 min at 4 °C, before resuspension in a desired volume of complete medium.

Pooled genome-wide CRISPR screen

To perform the whole-genome CRISPR screen, HepG2-CD81 cells were transduced with lentivirus containing the GeCKOv2 pooled sgRNA library of 123,411 sgRNAs targeting 19,031 protein-coding genes (~6 sgRNAs/gene), 1,864 microRNAs (4 sgRNA/microRNA) 1,000 negative controls (2 sgRNA/control), and selected in puromycin for 5-7 days. On day 12-14 post-transduction, 40 million puromycin-resistant cells were infected with GFP tagged-*P. yoelii* at a MOI of 0.3. After 24 h of infection, cells were sorted as infected and uninfected by FACS into different bins based on GFP signal. A non-treated, non-infected control was also collected for each experiment to assess library representation. The

experiment was performed four independent times. Genomic DNA from each sample was isolated using QIAamp DNA mini kit (Qiagen, Hilden, Germany).

Next-generation sequencing

Libraries were generated using a 2-step PCR according to previously published protocol (Sanjana et al., 2014). Briefly, an initial PCR was performed using AccuPrime Pfx Supermix (Invitrogen, Waltham, MA, USA) with lentiCRISPRv2 adaptor primers to amplify the sgRNA region and add priming sites for Illumina indexing. Amplicons were purified using FlashGels (Lonza, Allendale, NJ, USA) and purified PCR products were used as templates for subsequent PCR amplification. Sufficient PCR reactions were performed to maintain library coverage. Next, a second PCR was performed in order to add Illumina P5 and P7 index sequences, as well as barcodes for multiplexing, and samples were re-purified. Purified libraries were quantified using the KAPA library quantification kit (Kapa Biosystems, Wilmington, MA, USA) as per manufacturer's instructions performed on an Applied Biosystems 7500 Fast real-time PCR machine (Applied Biosystems, Foster City, CA, USA). Samples were sequenced on a MiSeq (Illumina) using the manufacturer's protocol with addition of Illumina PhiX control (Illumina, San Diego, CA, USA) to improve library diversity at a final concentration of 10% per library volume. After demultiplexing, FASTQ data files of 75bp single mate reads averaged 24.98 million raw reads per library.

Differential abundance of guides and gene enrichment analysis

FASTQ files were aligned to the GeCKOv2 pooled sgRNA library of 123,411 sgRNA DNA sequences by Bowtie2 (version 2.2.8) using local alignment policy command line arguments " --local -L 12 -N 0 -D 15 -i C,1,0 --gbar 8 --rdg 10,3 --rfg 10,3". This yielded

on average 20.41 million aligned reads to guides per library. Read counts per guide were converted to relative expression abundance as Reads Per Million (RPM). A guide was called detected in a screen if the before infection condition was at least 0.1 RPM. Undetected guides (RPM below 0.1) were excluded from further calculations. Fold change with respect to 'before infection' was calculated by dividing RPM in 'infected' or 'bystanders' conditions by RPM in 'before infection' condition. The differential abundance of a guide is represented as the $\log_2$ ratio of fold change in 'infected' condition divided by the fold change in 'bystanders' condition. If less than two screens call a guide detected (RPM >= 0.2), a $\log_2$FC of 0 and p-value of 1 are reported for this guide. Otherwise, the final $\log_2$FC of the guide is the arithmetic mean of the $\log_2$ ratios from each detected screen, and the final p-value of the guide is calculated by one sample t-test that the $\log_2$ ratios of the detected guides was not zero. The GeCKO library contains 6 independent guides for each protein-coding gene. The $\log_2$FC and p-value at the gene level is calculated from $\log_2$FC and p-value of its 6 guides. The $\log_2$FC of a gene is equal to the $\log_2$FC of the guide with the lowest (best) p-value. The corrected p-value of a guide is set to 1 if the sign of its $\log_2$FC is opposite to the $\log_2$FC of the gene. Then the p-value of a gene is calculated as the product of corrected p-values from all guides not excluded from calculations.

Gene set enrichment analysis on all genes with positive/negative $\log_2$FC was performed based on major knowledgebases including HUGO Gene Nomenclature Committee (HGNC), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome. The top 200 significantly enriched gene sets associated with all genes of negative or positive $\log_2$FC were identified. Genes that are both statistically significant (p-

value < 0.05) and differentially abundant (log_2FC < –6.0 or > 6.0) were considered significantly represented in *P. yoelii* infection. Go terms were clustered into higher order hierarchy using ClueGO plug-in (version 2.3.3), implemented in Cytoscape v3.4.0.

Generation of individual hits using gene specific CRISPR sgRNA

GFP tagged vectors for the 15 hits were obtained from ABM Good (Richmond, British Columbia, Canada). Non-replicating lentiviral stocks were generated by transfection of HEK293-FT cells. 4×10^6 HEK293-FT cells were plated on poly-L-lysine coated dishes to achieve 70-80% confluency at time of transfection. Approximately 24 h after plating, transfection mixtures were prepared by mixing 20 µl Polyethyleneimine MAX (Polysciences Inc, Warrington, PA) prepared at 1 mg/ml, together with 4.75 µg of sgRNA construct or a scramble control, along with 3rd generation lentiviral packaging mix from ABM Good, according to manufacturer's protocol. After incubating for 10 min at room temp in DMEM, transfection complexes were added dropwise to cells. After overnight incubation, cells were washed to remove transfection mixtures and were fed with 10 ml fresh media. Lentivirus-containing supernatant was harvested 36 hours later, passed through 0.45 µm syringe filters, and either used immediately for transduction or stored at -80 °C. To disrupt candidate genes, HepG2-CD81 cells were transduced with lentiviral supernatants in 6-well plates at a cell density of 1×10^6 per well. At time of plating, cells were transduced with 1 ml of supernatant in the presence of 0.5 µg/ml polybrene (Sigma Aldrich St. Louis, MO). In order to select for cells with stable integration of shRNA transgenes, supernatant was replaced with complete media with the addition of 2 µg/ml puromycin 24 h post-transduction, and cells were selected for at least 5 days prior to

experiments. For analysis of experiments with the knockout cells, only the GFP-positive cells have been considered.

Infection assay

5×10^5 HepG2-CD81 wild type cells or knockdown cells were seeded in each well of a 24-well plate (Corning) and infected with *P. yoelii* sporozoites at an MOI of 0.3 for 90 min and the infection was either stopped, or media was replaced and the infection was allowed to progress for 24, 36 or 48 h.

Flow cytometry

Cells were detached with accutase (Life technologies) and fixed with Cytoperm/Cytofix (BD Biosciences). Cells were blocked with Perm/Wash (BD Biosciences) + 2% (w/v) BSA for one hour at room temperature then stained overnight at 4 °C with Alexa Fluor -488 or -647 conjugated circumsporozoite (CSP) antibody. The cells were then washed and resuspended in PBS supplemented with 5 mM EDTA. Infection rates were measured by flow cytometry on an LSRII (Becton-Dickinson) and analyzed with FlowJo (Tree Star).

Immunofluorescence

For imaging experiments, HepG2-CD81 wild type or knockout cells were plated in 8 well chamber slides (Labtek) and infected with *P. yoelii* sporozoites. Cells were fixed with 3.7% (v/v) paraformaldehyde (Sigma) at defined timepoints after infection (90 min, 24 h, 36 h, or 48 h), permeabilized with Triton X-100, and stained with fluorescent tagged UIS-4 or HSP70 (70 kilodalton heat shock protein) antibodies. Nuclei were stained with DAPI (Vectashield). Images were acquired with a 100× 1.4 NA objective (Olympus) on a DeltaVision Elite High-Resolution Microscope (GE Healthcare Life Sciences). The sides

of each pixel represent 64.5 × 64.5 nm and z-stacks were acquired at 300 nm intervals. Approximately 20-30 slices were acquired per image stack. For deconvolution, the 3D data sets were processed to remove noise and reassign blur by an iterative Classic Maximum Likelihood Estimation widefield algorithm provided by Huygens Professional Software (Scientific Volume Imaging BV, The Netherlands). For the highthroughput secondary screen, cells were plated onto 96 well plate, infected and stained as explained above Images were acquired using Keyence BZ-X800 automated microscope and infection rate were quantified using Imaris 9.5, image analysis software.

Meta-analysis of screens

Z-scores of positively and negatively represented genes in each screen were calculated separately. Meta ranking was performed by function metaRank() from the R package DuffyTools, (using arguments: mode="percentile", rank.average.FUN=mean, naDropPercent = 0.75). Positively represented genes with z-scores greater than the cutoff (1.0, 1.5, 2.0) and negatively represented genes with z-scores smaller than the cutoff (-1.0, -1.5, -2.0) were selected as hits for each screen. Hits of positively and negatively represented genes were combined for further pathway enrichment analysis. To compare datasets of uneven sizes, gene rank percentiles were assigned to positively and negatively represented genes in each screen separately. Genes were ranked by the average percentiles across all datasets where they were screened.

Gene Ontology analysis on integrated forward genetic screens

Identified hit genes from all the four screens were uploaded in ious combinations in the ClueGO plug-in (version 2.3.3), implemented in Cytoscape v3.4.0 (http://cytoscape.org/) to generate gene ontology (GO) and pathway enrichment networks. Enriched functionally

annotated groups were obtained with the following setting parameters: organism was set to Homo sapiens; the total gene set used in each of the screen were used as reference; the gene ontology terms were accessed from the following ontologies/pathways: Biological Process and Reactome Pathway database evidence code was restricted to 'All_without_IEA'. The GO fusion option was also selected. The significance of each term was calculated with a two-sided hypergeometric test corrected with Benjamini-Hochberg correction for multiple testing. The kappa score was set to 0.5 and the GO tree levels were restricted at 6–16 (medium-detailed specificity). For GO term selection, a minimum of 3 genes and 3% coverage of the gene population was set. GO terms were grouped with an initial group size of 2 and 50% for group merge. The remaining parameters were set to defaults.

Statistical analysis

GraphPad Prism 7 was used for all statistical analyses. Statistical details for each experiment can be found in the figure legends, including the number of technical and biological replicates performed.

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Declaration of Interests

The authors declare no competing interests.

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Figure Legends

Figure 1. Whole genome hepatocyte CRISPR-Cas9 screen reveals putative *Plasmodium* liver stage regulatory factors. (A) Workflow of the hepatocyte CRISPR-Cas9 screen. A pooled CRISPR-Cas9 lentiviral sgRNA library is used to transduce HepG2-CD81 cells. Cells are infected with GFP-expressing *P. yoelli* sporozoites, and infected cells are isolated via FACS. Four biological samples were collected, and Illumina sequenced to quantify sgRNA counts from: uninfected cells, infected cells, and bystander cells. (B) sgRNAs observed at different levels in infected and uninfected cells are enriched in multiple GO biological processes. Nodes represent different biological processes, and the size of each node is scaled to the number of sgRNAs in the underlying gene set. Connection between nodes indicates that they share at least one gene. The nodes are grouped and further annotated. The nodes highlighted in blue are highly represented and characterized further in the study. GO terms are clustered based on higher order hierarchy using ClueGo cytoscape plugin. (C) Ranked $\log_2$ FC of genes with different levels of sgRNAs in infected and uninfected cells with a p-value < 0.05. Genes selected for further study are colored magenta ($\log_2$ FC > 0; negative regulators of infection) or green ($\log_2$FC < 0; positive regulators of infection). (D) A chord diagram of genes with significantly enriched sgRNAs in infected vs uninfected cells (p-value < 0.05, $\log_2$FC < -6.0 or > 6.0). An edge connecting genes indicates that they both belong to at least one gene set according to GO terms. Genes selected for further study are colored red ($\log_2$ fold change>0) or green ($\log_2$FC <0). Blue coded edges represent the connection between the genes belonging to the GO terms highlighted in (B).

Figure 2. Evaluation of selected hits from CRISPR-Cas9 screen for activity in LS infection and development. (A) HepG2-CD81 cells were transfected with CRISPR-Cas9 containing plasmids targeting the specified gene or scrambled control and challenged with *P. yoelii* sporozoites for 90 min. The bar graph depicts invasion as the rate of *Py*CSP-positive cells for each sgRNA knockout, normalized to a scrambled control. n=3; mean ± SD. Magenta represents negative regulators of infection and green represents positive regulators of infection. (B) HepG2-CD81 cells were pre-treated with or without the presence of specified compounds for 2 h and infected with *P. yoelii* sporozoites for 90 min. As in (A), the bar graph depicts the invasion rate. n=3; mean ± SD. (C) HepG2-CD81 cells were transfected with CRISPR-Cas9 containing plasmids targeting the specified gene or scrambled control and challenged with *P. yoelii* sporozoites. After 48 h, infection was evaluated using fluorescence microscopy. The bar graph depicts the infection rate after knockout of each transcript of interest normalized to scramble cells. n=3; mean ± SD. (D) HepG2-CD81 cells were infected with *P. yoelii* sporozoites for 90 min, washed and treated with or without the presence of specified compounds for 48 h. As in (C) the bar graph depicts the infection rate. n=3; mean ± SD (E) Assessment the Exo-erythrocytic forms (EEF) size from microscopic from (C). (n=3; mean ± SD). (F) A pie chart depicts HepG2-CD81 knockouts with significantly different entry, development or growth rates than the scrambled control. * Statistically significant at p-value < 0.05.

Figure 3. *P. yoelii*-infected cells exhibit altered Golgi morphology. (A, B) HepG2-CD81 cells were infected with *P. yoelii* sporozoites and the infection was allowed to proceed for 24 or 36 h. Cells were fixed and the parasite was visualized using an antibody against *Py*UIS4. The Golgi and Golgi derived vesicles were visualized with antibodies against FTCD (A) or GM130 (B). A representative in-focus single z-image is shown for (A) and maximum intensity projection of is shown for (B). The arrowhead shows the extending parasite tubulo-vesicular network localized in close association with the Golgi marker. Bar = 2 µm. Images are representative of 45 images from three independent experiments.

Figure 4. *P. yoelii*-infected cells exhibit alterations in microtubule organization and positioning. (A) HepG2-CD81 cells transfected with CellLight™ RFP-α-Tubulin BacMam 2.0 were infected with *P. yoelii* sporozoites for 24 h. Cells were fixed and stained with antibodies to *P. yoelii* HSP70 and acetylated α-tubulin. Images are maximum intensity projections. (B) HepG2-CD81 cells transfected with CellLight™ GFP-α-Tubulin BacMam 2.0 were infected with *P. yoelii* sporozoites for 24 h. Cells were fixed and stained with antibodies to *P. yoelii* UIS4 and CAMSAP2. Images shown are single representative in-focus z-slice. (C) HepG2-CD81 and HepG2-CD81-sgRNA-CENPJ cells were infected with *P. yoelii* sporozoites for 24 h. Cells were fixed and stained with antibodies against *P.yoelii* UIS4-647 conjugate (pseudo colored-green) and γ-tubulin to visualize the parasite PVM and the microtubule organizing center (MTOC), respectively. Images shown are single representative z-stack from 20-25 slices. A pie chart showing localization of MTOC in the cell during different conditions. Bar = 2 µm.

Figure 5. Meta-analysis of *Plasmodium* liver stage screens reveals areas for future investigation. (A) A Venn diagram showing gene-level coverage in three screening efforts. (B) A Venn diagram depicts gene-level overlap of the negative regulators of infection identified following meta-analysis (Supplementary file 1) on the gene hits from three screens compared with the z-score cut-off of 2, 1.5 and 1. (C) A Venn diagram showing overlap of positive regulators of infection identified following meta-analysis (Supplementary file 1) on the gene hits from the three screens compared with the z-score cut-off of 2, 1.5 and 1. (D) Network analysis on the enriched GO biological processes predicted from significantly enriched genes identified from at least two of three screens with the z-score cut-off of 1.5. Nodes represent biological processes, interactions between pathways. The gene and pathways identified from the analysis shared by the biological processes are represented solid arrows while the gene shared by the biological processes are represented as dashed lines. The significant biological processes identified from the network analysis (D) are color coded in (E) and tabulated with corresponding p values.

Figure S1: (A) HepG2-CD81 cells were transfected with CRISPR-Cas9 - GFP containing plasmids targeting the specified gene or scrambled control. A representative histogram shows levels of GFP post transfection. (B) Western blot with anti-CENPJ antibody showing the expression levels of CENPJ in scrambled and CENPJ knockout cells. (C) The bar graph represents the quantification of CENPJ expression in wildtype and CENPJ knockout cells. n=3; mean ± SD. (D) Evaluation of cytotoxicity profile of small molecules used in the study. Data are presented as the mean cytotoxicity value ± standard deviation from one representative experiment of three independent experiments.

Supplementary table 1: Inhibitors used in the study. Related to figure 2.

Inhibitor	Function	Reference
NL-1	mitoNEET Inhibitor (Gene: CISD1)	(Geldenhuys et al., 2014)
HPA-12	CERT antagonist (Gene: COL4A3BP)	(Santos et al., 2015)
SMFH2	Small molecule inhibitor of formin homology 2	(Kim et al., 2015)
Cisplatin	Perturbs iron metabolism by inhibiting IREB2	(Miyazawa et al., 2019)
LDLR peptide	Blocks LDL receptor function	(Ye et al., 2014)
CP91149	selective glycogen phosphorylase (GP) inhibitor	(Martinez-Navarro et al., 2020)
NSC-697923	Ubiquitin E2 Conjugating enzyme inhibitor	(Pulvino et al., 2012)
Eltanexor	Second generation XPO1 inhibitor	(Luedtke et al., 2018)

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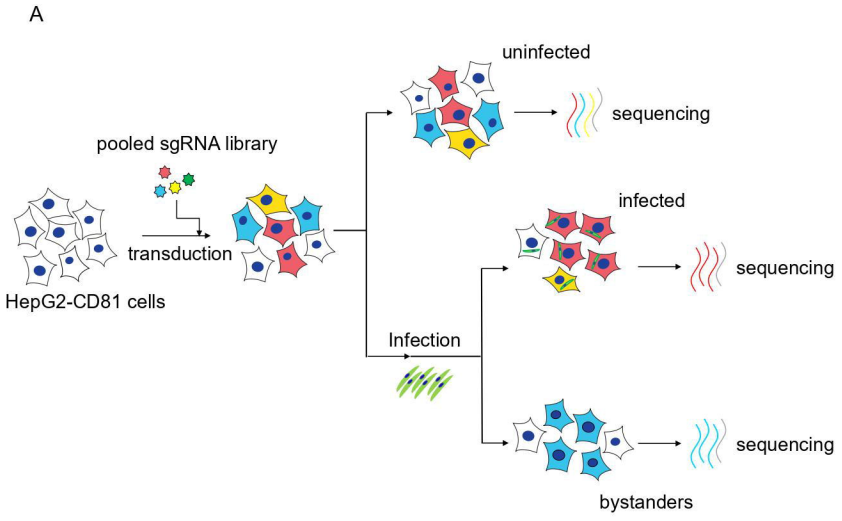
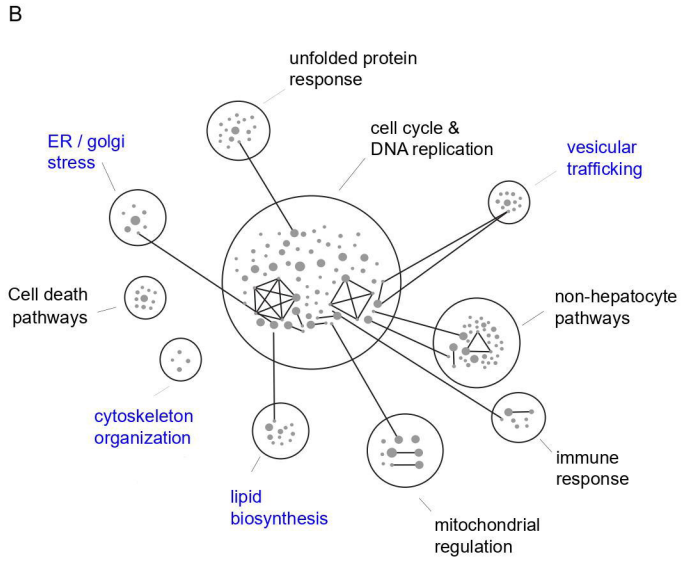
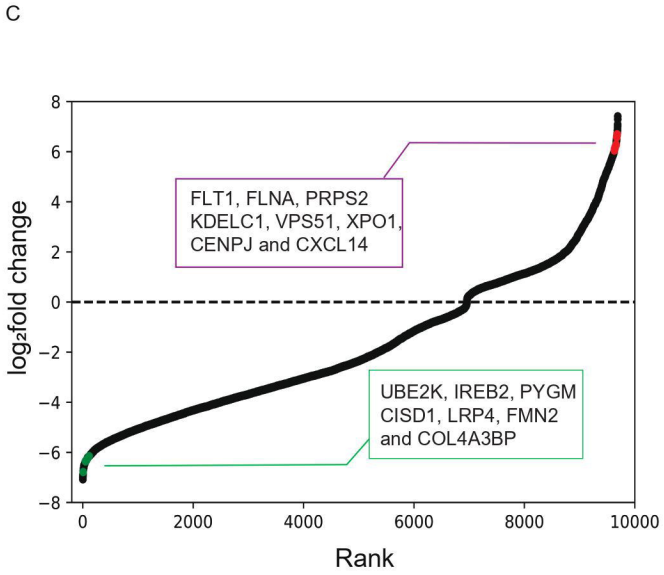
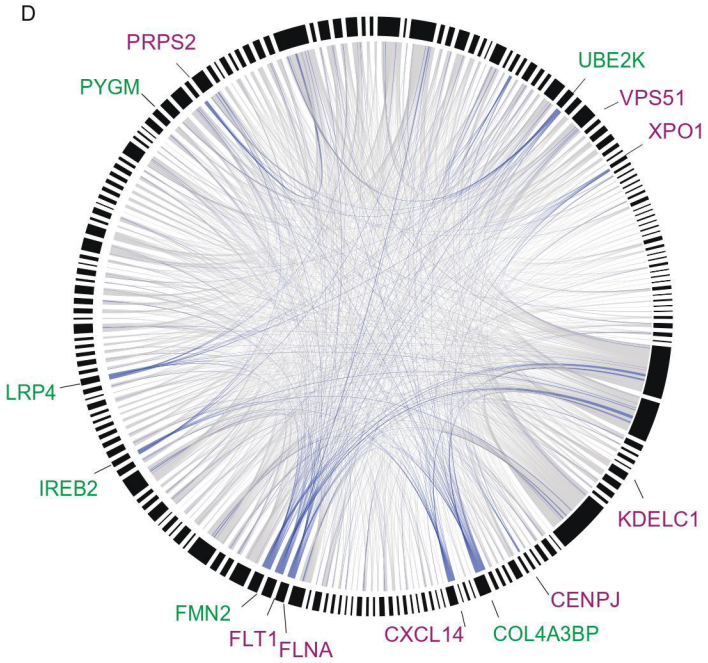
Supplementary Table 2. Comparison of our CRISPR/Cas9 screen with previous host-based genetic intervention screens. Related to figure 5.

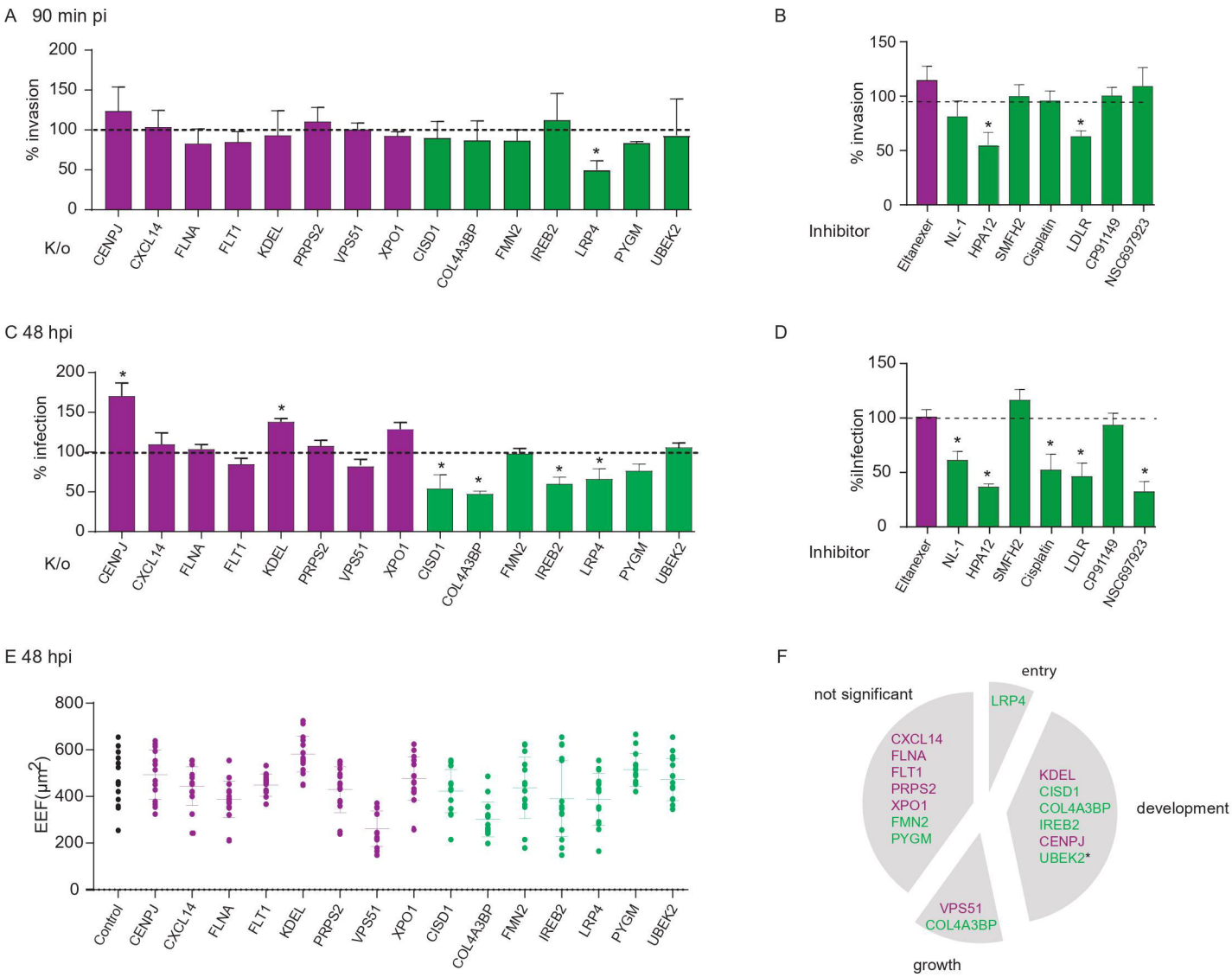
Reference	Coverage	Species	Method	Cells	Identified hits
Rodrigues et al., 2008	Host lipoprotein pathway (53 genes)	*P. berghei*	RNAi	Huh7	1
Prudencio et al., 2008	Kinome-wide (727 genes)	*P. berghei*	RNAi	Huh7	16
Raphemot et al., 2019	Human druggable genome (6951 genes)	*P. berghei*	RNAi	HepG2 and Huh7	177
Vijayan et al. (this study)	Genome-wide (21775 genes)	*P. yoelii*	Pooled CRISPR/Cas9	HepG2-CD81	242


A
uninfected
sequencing
pooled sgRNA library
infected
transduction
sequencing
HepG2-CD81 cells
Infection
sequencing
bystanders

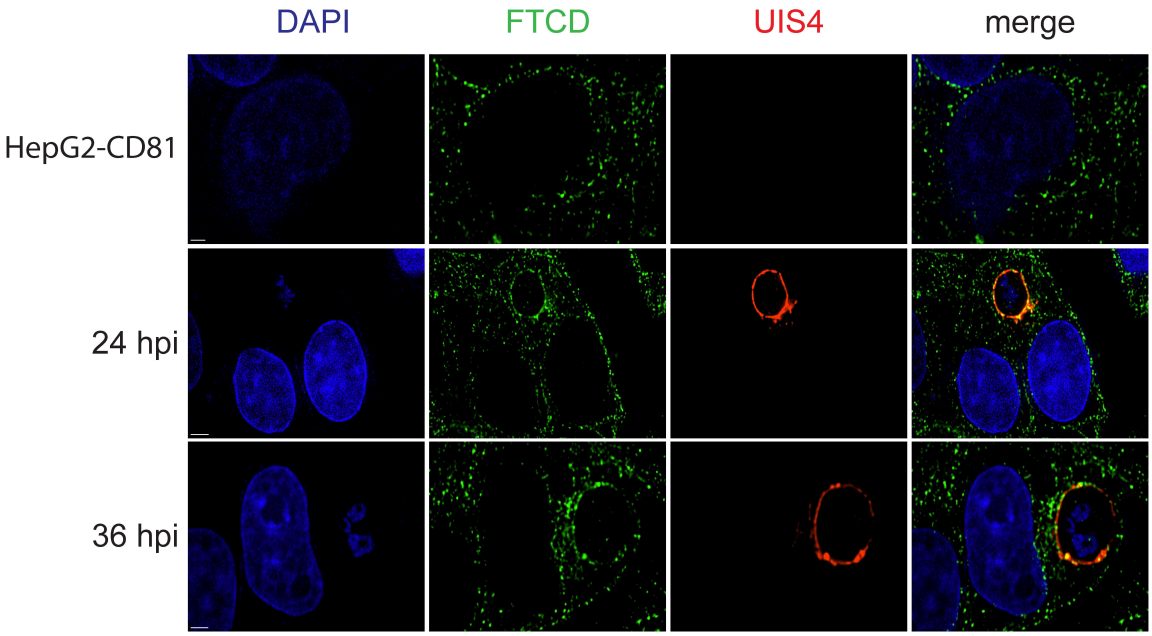

B
unfolded protein response
ER / golgi stress
cell cycle & DNA replication
vesicular trafficking
Cell death pathways
non-hepatocyte pathways
cytoskeleton organization
immune response
lipid biosynthesis
mitochondrial regulation


C
FLT1, FLNA, PRPS2
KDELC1, VPS51, XPO1,
CENPJ and CXCL14
UBE2K, IREB2, PYGM
CISD1, LRP4, FMN2
and COL4A3BP
log2fold change
Rank
8
6
4
2
0
−2
−4
−6
−8
0
2000
4000
6000
8000
10000

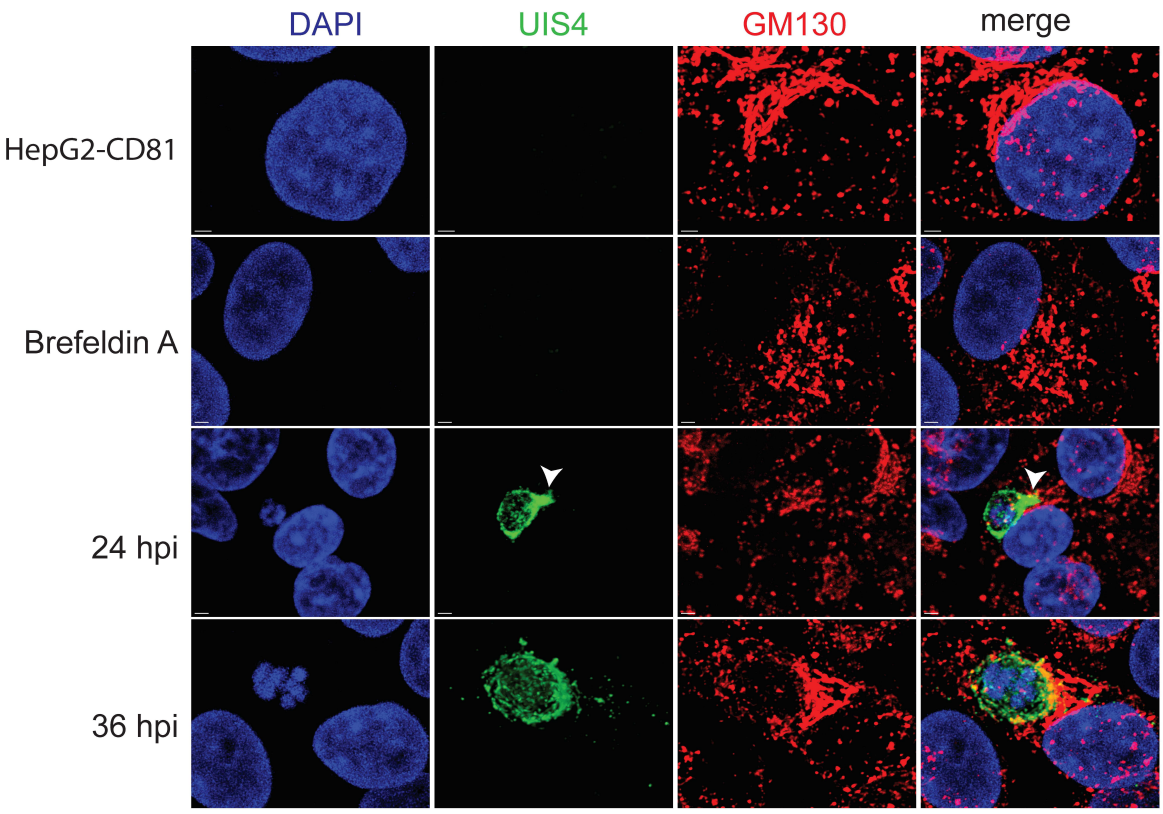

D
PRPS2
PYGM
UBE2K
VPS51
XPO1
LRP4
IREB2
KDELC1
CENPJ
FMN2
FLT1
FLNA
CXCL14
COL4A3BP


A 90 min pi
% invasion
K/o
CENPJ
CXCL14
FLNA
FLT1
KDEL
PRPS2
VPS51
XPO1
CISD1
COL4A3BP
FMN2
IREB2
LRP4
PYGM
UBEK2
B
% invasion
Inhibitor
Eltanexer
NL-1
HPA12
SMFH2
Cisplatin
LDLR
CP91149
NSC697923
C 48 hpi
% infection
D
%iInfection
E 48 hpi
EEF(µm2)
Control
F
entry
not significant
LRP4
CXCL14
FLNA
FLT1
PRPS2
XPO1
FMN2
PYGM
KDEL
CISD1
COL4A3BP
IREB2
CENPJ
UBEK2*
development
VPS51
COL4A3BP
growth

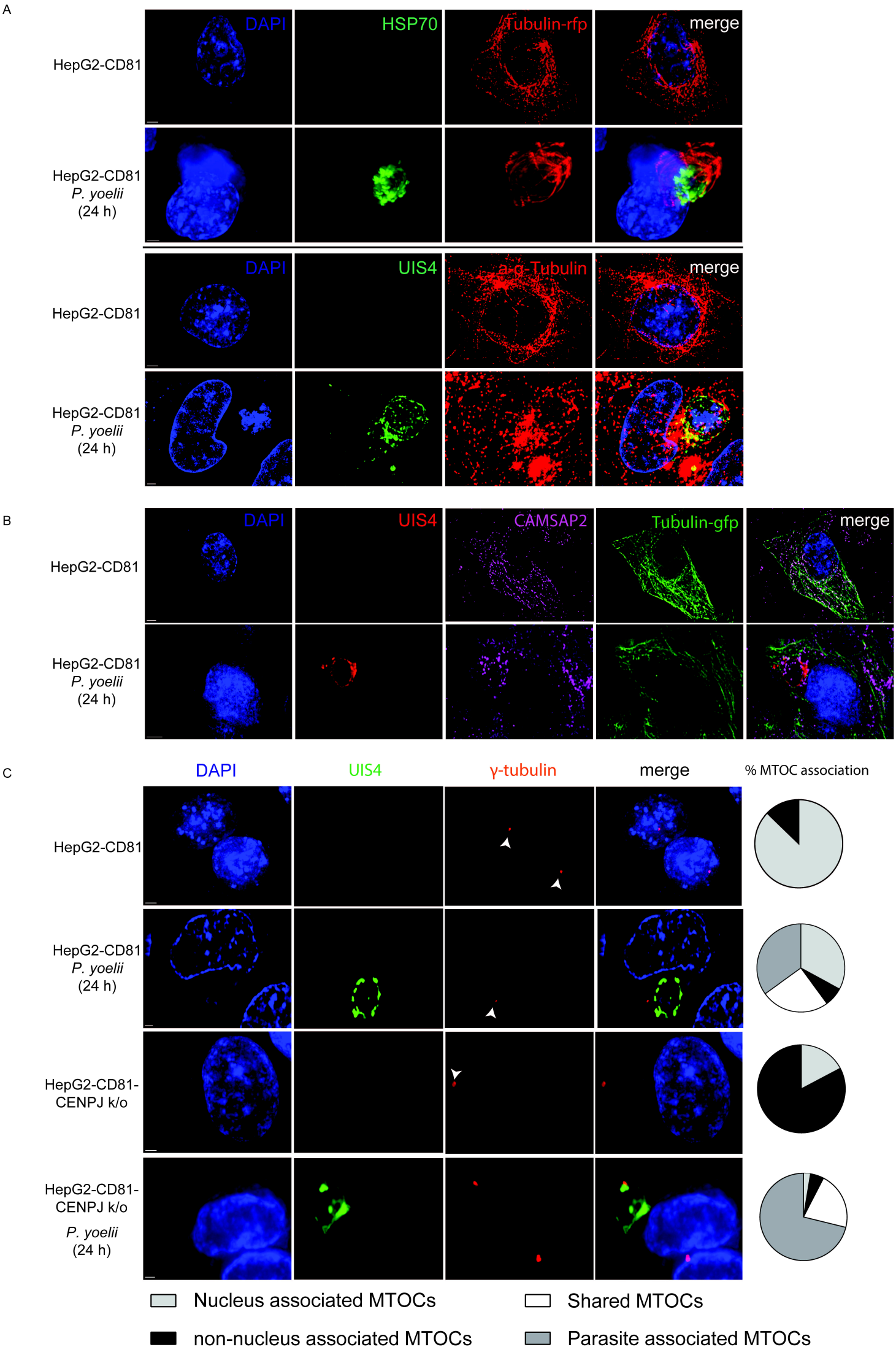
A



B

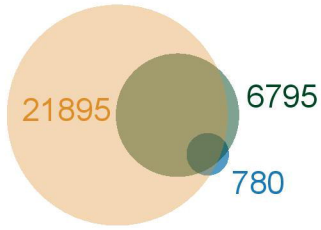


	% cells with intact Golgi	% cells with PVM-Golgi contact
HepG2-CD81	86 ± 5	0
Brefeldin A	12 ± 4	0
24 hpi	27 ± 6	75 ± 9
36 hpi	70 ± 6	93 ± 3


A
DAPI
HSP70
Tubulin-rfp
merge
HepG2-CD81
HepG2-CD81
P. yoelii
(24 h)
DAPI
UIS4
a-g-Tubulin
merge
HepG2-CD81
HepG2-CD81
P. yoelii
(24 h)
B
DAPI
UIS4
CAMSAP2
Tubulin-gfp
merge
HepG2-CD81
HepG2-CD81
P. yoelii
(24 h)
C
DAPI
UIS4
γ-tubulin
merge
% MTOC association
HepG2-CD81
HepG2-CD81
P. yoelii
(24 h)
HepG2-CD81-
CENPJ k/o
HepG2-CD81-
CENPJ k/o
P. yoelii
(24 h)
Nucleus associated MTOCs
Shared MTOCs
non-nucleus associated MTOCs
Parasite associated MTOCs

A

Screen coverage



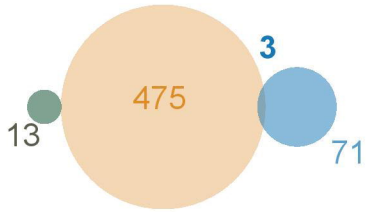
Our Screen

Raphemot et al., 2019

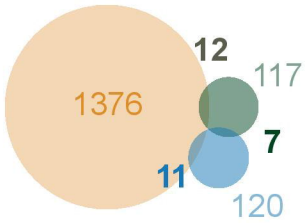
Rodrigues et al., 2008 and Prudencio et al., 2008

B

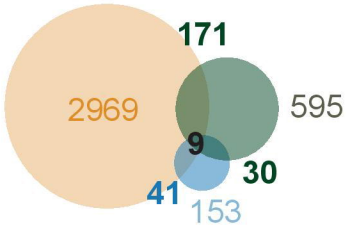
Negative regulators of infection



Z score: 2



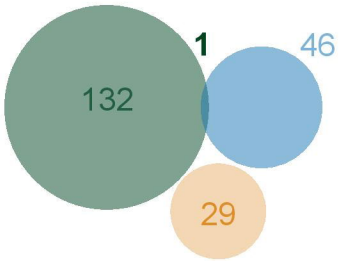
Z score: 1.5



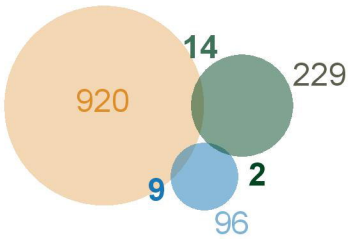
Z score: 1

C

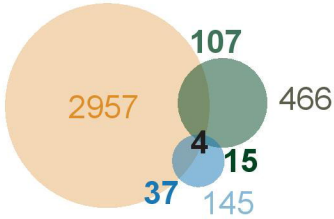
Positive regulators of infection



Z score: 2

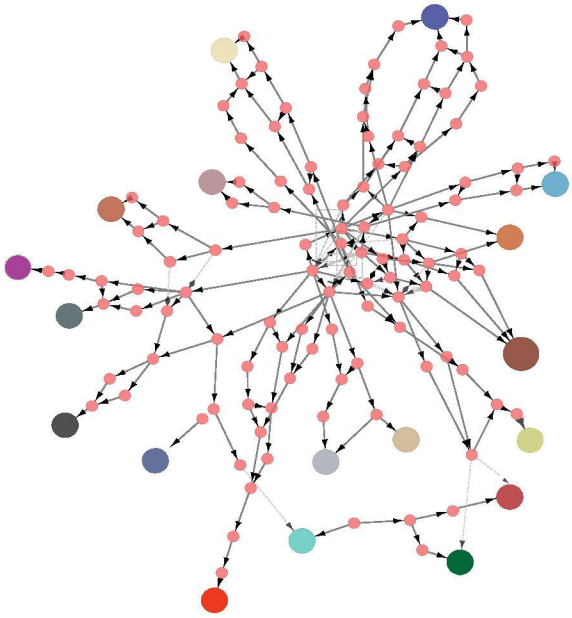


Z score: 1.5



Z score: 1

D

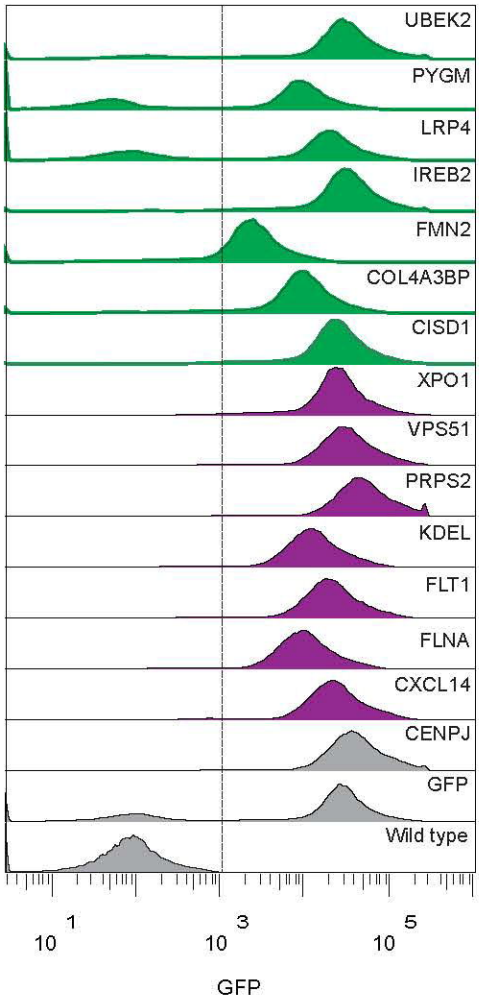


Z score: 1.5

E

pathways enriched in two or more screens	pvalue
positive regulation of heterotypic cell-cell adhesion	0.01305
regulation of insulin signaling pathway	0.03117
positive regulation of cytokine-mediated signaling pathway	0.00378
activation of JUN kinase activity	0.04123
MAP kinase kinase kinase activity	0.03893
MAP kinase kinase activity	0.01621
carbohydrate kinase activity	0.03653
negative regulation of axonogenesis	0.00654
anterograde cargo transport	0.02558
scavenger receptor activity	0.01798
triglyceride biosynthetic process	0.03824
canonical glycolysis	0.03374
glycogen metabolic process	0.01545
regulation of cholesterol biosynthetic process	0.02558
positive regulation of protein localization to plasma membrane	0.02352
cellular response to amyloid-beta	0.03893
microgilial cell activation	0.03824
actrocyte activation	0.01784

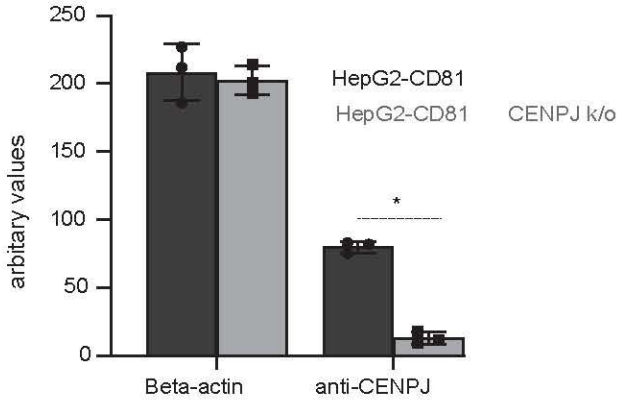
A


UBEK2
PYGM
LRP4
IREB2
FMN2
COL4A3BP
CISD1
XPO1
VPS51
PRPS2
KDEL
FLT1
FLNA
CXCL14
CENPJ
GFP
Wild type
10^1
10^3
10^5
GFP

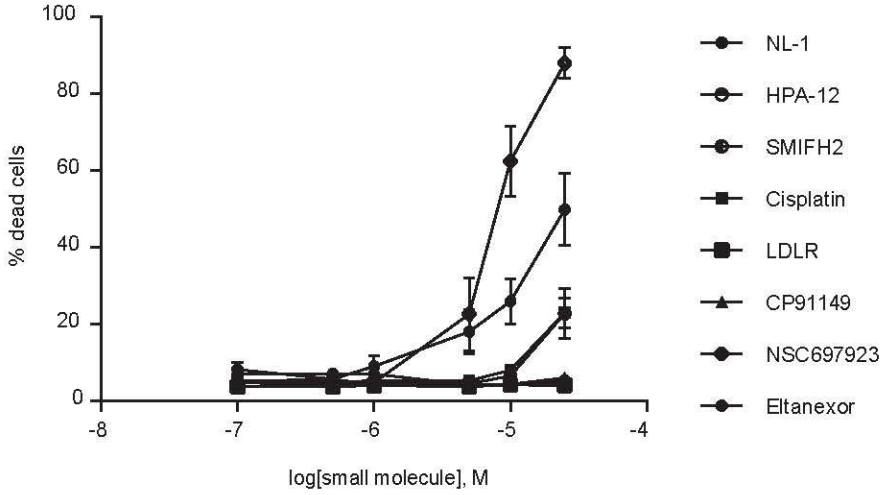
B

HepG2-CD81 scramble
HepG2-CD81 CENPJ k/o
153 kDa
42 kDa
anti-CENPJ
beta actin

C


250
200
150
100
50
0
arbitary values
HepG2-CD81
HepG2-CD81 CENPJ k/o
*
Beta-actin
anti-CENPJ

D


100
80
60
40
20
0
% dead cells
-8
-7
-6
-5
-4
log[small molecule], M
NL-1
HPA-12
SMIFH2
Cisplatin
LDLR
CP91149
NSC697923
Eltanexor