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Human cytomegalovirus protein RL1 degrades the antiviral factor SLFN11 via recruitment of the CRL4 E3 ubiquitin ligase complex

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Abstract

Human cytomegalovirus (HCMV) is an important human pathogen and a paradigm of viral immune evasion, targeting intrinsic, innate and adaptive immunity. We have employed two novel, orthogonal multiplexed tandem mass tag-based proteomic screens to identify host proteins downregulated by viral factors expressed during the latest phases of viral infection. This approach revealed that the HIV-1 restriction factor Schlafen-11 (SLFN11) was degraded by the poorly characterised, late-expressed HCMV protein RL1, via recruitment of the Cullin4-RING E3 Ubiquitin Ligase (CRL4) complex. SLFN11 potently restricted HCMV infection, inhibiting the formation and spread of viral plaques. Overall, we show that a restriction factor previously thought only to inhibit RNA viruses additionally restricts HCMV. We define the mechanism of viral antagonism and also describe an important resource for revealing additional molecules of importance in antiviral innate immunity and viral immune evasion.

Significance Statement

Previous proteomic analyses of host factors targeted for downregulation by HCMV have focused on early or intermediate stages of infection. Using multiplexed proteomics, we have systematically identified viral factors that target each host protein downregulated during the latest stage of infection, after the onset of viral DNA replication. Schlafen-11 (SLFN11), an interferon-stimulated gene and restriction factor for retroviruses and certain RNA viruses, potently restricted HCMV infection. Our discovery that the late-expressed HCMV protein RL1 targets SLFN11 for proteasomal degradation provides the first evidence for a viral antagonist of this critical cellular protein. We therefore redefine SLFN11 as an important factor that targets DNA viruses as well as RNA viruses, offering novel therapeutic potential via molecules that inhibit RL1-mediated SLFN11 degradation.

Main Text

Introduction

Human cytomegalovirus (HCMV) is a ubiquitous pathogen that establishes a lifelong latent infection in the majority of the world's population (1). Reactivation from latency in immunocompromised individuals, such as transplant recipients and AIDS patients, can result in significant morbidity and mortality (2). HCMV is also the leading cause of infectious congenital birth defects, including deafness and intellectual disability, affecting ~1/100 pregnancies (1). However, only a few antiviral drugs are approved for the treatment of HCMV, all of which are associated with significant toxicity, and there is currently no licensed vaccine (3).

Susceptibility to viral infection and disease is determined in part by antiviral restriction factors (ARFs) and the viral antagonists that have evolved to degrade them (4). Small molecules that inhibit ARF-antagonist interactions may restore endogenous restriction and offer novel therapeutic potential (5). Identification of novel ARFs and characterisation of their interactions with HCMV antagonists is therefore clinically important.

HCMV possesses the largest human herpesvirus genome, encoding 170 canonical open reading frames (ORFs). A modest number of non-canonical ORFs may encode additional functional proteins (6-9). During productive HCMV infection, viral gene expression occurs in cascades during a ~96 h infection cycle that is conventionally divided into immediate-early, early and late phases. Early genes encode functions necessary for initiating viral DNA replication. In the late phase, early-late genes are initially transcribed at low levels and are then upregulated after the onset of viral DNA replication, whereas true-late genes are expressed exclusively after DNA replication commences and include proteins required for HCMV virion assembly. We previously characterised five temporal classes of viral protein expression, offering finer definition of protein expression profiles (10).

As over 900 proteins are downregulated more than three-fold during the course of HCMV infection, predicting molecules likely to perform novel immune functions is challenging without additional data (7, 10, 11). Our previous analysis of the subset of proteins targeted for degradation by 24 or 48 h led directly to the identification of Helicase-Like Transcription Factor (HLTF) as a novel ARF, and HCMV UL36 as a key inhibitor of necroptosis, by degrading Mixed Lineage Kinase-domain-Like protein (MLKL) (7, 10).

However, no studies have systematically examined which host factors are targeted by viral proteins during the latest phase of infection. This question is important as some host factors may play important roles in restricting the final stages of viral replication. Furthermore, despite our prior characterisation of a comprehensive HCMV interactome (9), the abundance of certain host proteins whose expression is downregulated during infection can be sufficiently low to impede identification of their viral antagonists.

We have used two complementary proteomic approaches to address these questions. The first identified cellular proteins specifically targeted by HCMV factors expressed after viral DNA replication, by comparing host protein expression over time in the presence or absence of the viral DNA polymerase inhibitor phosphonoformic acid (PFA). The second employed an enhanced panel of HCMV mutants each deleted in contiguous gene blocks dispensable for virus replication *in vitro*, most of which we have described previously (12).

The intersection between these approaches showed that one particular protein, Schlafen family member 11 (SLFN11), is both downregulated during the late phase of HCMV infection and is targeted by the RL1-6 block of viral genes. SLFN11 potently restricted HCMV infection and therefore represents a novel HCMV ARF. Among the factors encoded by the RL1-6 region, RL1 was required for SLFN11 downregulation, via recruitment of the Cullin4-RING E3 Ubiquitin Ligase (CRL4) complex. Overall, our data identifies a novel HCMV ARF and a novel mechanism of viral antagonism, and describes an important resource that will reveal additional molecules of importance in antiviral innate immunity and viral immune evasion.

Results

Host proteins downregulated by late-expressed HCMV factors

To globally quantify cellular proteins whose expression is increased or decreased by late-expressed HCMV factors, we applied PFA to HCMV-infected primary human fetal foreskin fibroblasts (HFFFs) at the time of infection and harvested samples for analysis at 24h intervals (**Figure 1A**). Expression of early viral genes is largely unaffected by PFA, whereas early-late genes are partially inhibited and late genes are completely inhibited (13). Ten-plex tandem mass tag (TMT) technology and MS/MS/MS mass spectrometry of whole-cell lysates enabled precise protein quantification (**Figure 1A**).

We quantified 8059 human and 149 viral proteins, and observed good correspondence between proteins modulated during HCMV infection in the absence of PFA and protein expression in our previously published proteomic datasets (10) (**Figure S1**). Overall, by 96 hours post infection (hpi), 157 human proteins were downregulated ≥3-fold in the absence of PFA and 'rescued' >2-fold in the presence of PFA (**Figure 1B, Dataset S1A**). Application of DAVID software (14) indicated that these included groups of plasma membrane proteins, proteins with immunoglobulin or cadherin domains, and proteins with functions in viral infection (**Figure S2A, Dataset S1B**). Examples included multiple collagens, ephrins, syndecans and adhesion molecules such as junctional adhesion molecule-3 (JAM3), in addition to T-cell co-stimulator CD276 and DNA replication inhibitor and HIV-1 restriction factor Schlafen-11 (SLFN11) (15, 16) (**Figure 1C**). Additionally, 87 human proteins were both upregulated ≥3-fold by 96 hpi yet downregulated >2-fold in the presence of PFA (**Figures S2B-C, Dataset S1C**), indicating that late-expressed viral proteins can exhibit additional functions in host regulation.

RL1 is necessary and sufficient for SLFN11 downregulation

Identification of which HCMV protein(s) target a given cellular factor can be challenging due to the substantial coding capacity of HCMV. To identify viral proteins targeting host factors late during HCMV infection, we extended our previous approach that analysed infection at 72 h with a panel of recombinant viruses, each deleted for one or other of a series of blocks of genes non-essential for replication *in vitro* (12) (**Figure S3A**). In this analysis, all viruses were examined in at least biological duplicate, and for the first time ΔRL1-6 HCMV was included since the functions of HCMV factors encoded within this gene block (RL1, RL5A, RL6 proteins and the RNA2.7 long non-coding RNA) are poorly characterised. For each human protein, a z-score and fold change (FC) compared to wild-type (wt) infection was calculated

(see SI Methods). Sensitive criteria with a final z-score of >4 and FC >1.5 assigned 254 modulated cellular proteins to viral blocks (**Figure 2A**), and stringent criteria (z-score>6, FC>2) assigned 109 proteins to viral blocks (**Figure S3B, Dataset S2**). Data from this and the PFA screens are shown in **Dataset S3**, where the worksheet ‘‘Plotter’’ is interactive, enabling generation of graphs of expression of any of the human and viral proteins quantified.

To identify host factors targeted for downregulation by late-expressed HCMV proteins, data from the PFA and gene-block screens were combined. Using sensitive criteria, 17 host proteins were downregulated ≥3-fold by 96 hpi, ‘rescued’ >2-fold by PFA and targeted by one or other of the viral gene blocks examined (**Figure 2B**). These included proteins with previously described HCMV protein antagonists, for example known targets of the US18-US22 block including ALCAM, CD276 and JAM3, and PTPRM, which is a target of the US12-US17 block (**Figures 2B-C**) (17). The only assigned target of the RL1-RL6 block that met the threshold for rescue by PFA was SLFN11 (**Figure 2C**). Furthermore, of the proteins that targeted this block, SLFN11 was the most substantially modulated (**Figure S3C**).

To determine which viral protein targets SLFN11 for downregulation, C-terminally V5-tagged RL1, RL5A and RL6 constructs were stably overexpressed in HFFFs immortalised with human telomerase (HFFF-TERTs). Overexpression of RL1-V5 alone was sufficient for downregulation of SLFN11 and this was recapitulated by transient transfection of HEK-293 cells with RL1-V5 (**Figure 2D**). Furthermore, RL1 was necessary for downregulation of SLFN11 in the context of infection, since neither a single-gene Merlin RL1-deletion mutant nor the RL1-RL6 block deletion recombinant were able to reduce SLFN11 levels (**Figure 2E**). During HCMV infection, expression of RL1 was completely inhibited by the addition of PFA, and the profile of RL1 expression inversely correlated with the profile of SLFN11 (**Figures 1C, 2F, S4**).

RL1 degrades SLFN11 through recruitment of the Cullin4 E3 Ligase Complex

The HCMV RL1 and UL145 genes are related to each other and thus belong to the RL1 family (6). We and others have previously shown that the UL145 protein can employ CRL4 complex components CUL4A and DDB1 to degrade HLTF and STAT2 (7, 18). Using SILAC immunoprecipitation and co-immunoprecipitation, we identified a similar interaction between RL1 and DDB1 and CUL4A (**Figures 3A-B, Dataset S4**). A panel of alanine substitution mutations was tested to identify the region within RL1 required for interaction with DDB1 based on the DDB1 interaction motif previously identified within UL145 (19) (**Figure 3C**). As predicted, residues LL153-4, R157 and R159 were required for DDB1

binding, whereas residue T152 was dispensable. In contrast to residue N25 in UL145, which is indispensable for binding DDB1, the equivalent residue P149 in RL1 was not required. This may reflect the differences in the chemical properties of proline and asparagine residues, or the conservation within the DCAF family of asparagine at this position. Residues LL153-4, R157 and R159 are completely conserved across all publicly available HCMV RL1 sequences (263 different strains), and the corresponding residues in HCMV UL145 are also completely conserved (264 different strains). Furthermore, the LLxxRxR motif is highly conserved (complete conservation in 7/8 RL1 orthologues and 8/8 UL145 orthologues, **Figure S5**).

To determine whether the CRL4 complex is required for RL1-mediated degradation of SLFN11, components of the complex were knocked down in HFFF-TERTs stably expressing RL1 or control. Knockdown of DDB1 and CUL4A/4B prevented RL1-mediated loss of SLFN11 (**Figures 3D and S6A**). These results were recapitulated in the context of HCMV infection (**Figure 3E and S6B**). SLFN11 was also rescued from degradation in the presence of MLN4924, which prevents the conjugation of NEDD8 on cullins (20), substantiating the requirement for the CRL4 complex in RL1-mediated SLFN11 degradation (**Figure 3F**). This suggests that RL1 may redirect the Cullin 4 ligase complex to degrade SLFN11, by acting as a viral DDB1-Cullin Accessory Factor (DCAF).

SLFN11 restricts HCMV infection

We sought to determine whether SLFN11 restricts HCMV infection. SLFN11 depletion consistently and significantly increased HCMV replication in 4/4 independent HFFF-TERT cell lines stably knocked down for SLFN11, in terms of both number and size of plaques (**Figures 4A-B**). A decrease in the number of plaques was observed upon SLFN11 overexpression (**Figure 4C**). Multi-step growth curves of both RL1-replete and RL1-deficient viruses confirmed a relative replication defect in SLFN11-deficient cells (**Figures 4D-E**). A greater effect was observed at lower MOI as we and others have noted during the characterization of other antiviral restriction factors (7). SLFN11 therefore represents a novel ARF for HCMV that acts to restrict significantly the spread of HCMV infection.

Discussion

HCMV and other herpesviruses comprehensively modulate adaptive and innate immunity to facilitate their persistence, employing multiple viral proteins to target cellular factors for degradation (7). Although some viral proteins are expressed throughout the course of infection, others are temporally controlled and target a given host factor at a specific phase of viral replication (7, 11). The present study provides a systematic, searchable database that examines host protein regulation from the point of replication of the viral genome onwards, in addition to identifying which viral gene block targets each of >250 host factors.

The key roles of ARFs in protecting cell populations against HCMV are highlighted by the diversity of proteins with antiviral activity, with different factors affecting distinct steps of the HCMV replication cycle (reviewed in Schilling et al. (23)). Since description of protein components of promyelocytic leukemia bodies (PML, Sp100, hDaxx) as anti-HCMV ARFs, at least 15 additional ARFs have been identified, including HLTF, Zinc finger Antiviral Protein (ZAP), the cytidine deaminase APOBEC3A and the dNTP triphosphohydrolase SAMHD1. Some of these proteins exhibit antiviral activity against diverse viruses, whereas others, such as HLTF, have so far only been associated with restriction of HCMV.

We have now identified SLFN11 as a novel HCMV restriction factor, although the mechanism of restriction is yet to be determined. SLFN11 inhibits replication of lentiviruses in a codon usage-dependent manner, via its activity as a type II tRNA endonuclease (15, 16, 24, 25). Overall, HCMV genomes exhibit low codon usage bias, although the bias of individual coding sequences varies widely (26). Hu et al. (26) previously determined HCMV codon usage bias on a gene-by-gene basis. However, an analysis of their data using our temporal classification of HCMV protein expression (10) suggested that there is no systematic temporal codon usage bias of HCMV genes. It is possible that RL1-mediated SLFN11 degradation is required for efficient translation of certain poorly codon-optimised late-expressed viral genes. However, expression of poorly codon-optimised early-expressed viral genes would presumably still be reduced irrespective of RL1 expression. SLFN11 also inhibits translation of certain poorly codon-optimised human genes in the presence of DNA damaging agents, in particular genes specifying the serine/threonine kinases ATM and ATR (27). Both play key roles in the DNA damage response. HCMV requires ATM signaling for efficient replication, although the role of ATR signaling is presently unclear (reviewed in (28)). RL1 might thus prevent SLFN11-mediated repression of ATM/ATR in the presence of the DNA damage response stimulated by HCMV infection in order to benefit viral replication. Further alternative mechanisms are suggested by the recent identification of SLFN5 as an ARF for herpes simplex virus 1 (HSV-1) and SLFN14 as an ARF for influenza virus. SLFN5 interacts with HSV-1 viral DNA to

245 repress HSV-1 transcription (29), whereas SLFN14 promotes a delay in viral nucleoprotein translocation
246 from cytoplasm to nucleus and enhances RIG-I mediated IFN-β signaling (30). These observations
247 suggest that other components of the six-member human Schlafen family may act as restriction factors for
248 HCMV, and that Schlafen proteins may more widely restrict other DNA and RNA viruses. Indeed, we
249 found that SLFN5 was downregulated early during HCMV infection (Dataset S3), raising the intriguing
250 possibility that the virus differentially regulates members of this important family to maximise viral
251 replication.

252 Several viruses are now recognised to encode factors that degrade host protein targets by subverting
253 cullins or their adaptor proteins, including hepatitis B, HIV, parainfluenza virus, bovine herpesvirus,
254 murine gammaherpesvirus and CMVs (reviewed in (31, 32)). Including RL1, four CMV proteins have
255 now been recognized to function in this manner, all via recruitment of CRL4 components: murine CMV-
256 encoded M27 and HCMV-encoded UL35 and UL145 (7, 33-35). However, in our recent comprehensive
257 HCMV interactome analysis (9), we detected six additional HCMV proteins that interact with CUL4A or
258 CUL4B (RL12, US7, US34A, UL19, UL122 and UL135), two additional proteins interacting with DDB1
259 (UL19 and UL27), and three viral proteins interacting with other cullins (US30, UL26 and UL36). These
260 data suggest that there are likely to be additional as yet uncharacterized mechanisms for HCMV-mediated
261 cullin subversion, which may lead to degradation of additional host targets.

262 The presence of orthologs of RL1 and UL145 in the same positions and orientations in Old and New
263 World monkey and ape cytomegalovirus genomes indicates that this pair of genes has existed for at least
264 40 million years. Furthermore, the conservation of amino acid residues required for DDB1 interaction
265 suggest that the functions they serve are both ancient and essential for viral replication. Presumably, one
266 or other of these genes developed first (perhaps by a now undetectable gene capture) and then duplicated.
267 Sequences from early primate branches would be required to investigate the evolutionary history further,
268 but these are presently lacking.

269 Our identification of RL1-mediated SLFN11 degradation provides the first evidence for direct viral
270 antagonism of this important restriction factor, and might help to explain the evolution of SLFN11 under
271 recurrent positive selection throughout primate development (25). Other mechanisms may also underlie
272 this selection. Schlafen genes acquired by orthopoxviruses might inhibit their host counterparts, possibly
273 by preventing cellular Schlafen multimerisation (25, 36). Certain flaviviruses might also encode anti-
274 SLFN11 mechanisms, which could explain the differential susceptibility of West Nile, Zika and dengue
275 viruses to SLFN11 effects (37). Additionally, sperm-egg interactions and meiotic drive can both result in

strong signatures of recurrent positive selection, and some mammalian Schlafen genes have been implicated in sperm-egg incompatability (25, 36).

Only three drugs are commonly used in HCMV treatment, all exhibiting significant adverse effects and the risk of drug resistance. A novel therapeutic approach would be to prevent interaction of virally encoded immune antagonists with their cellular partners. The interaction of RL1 with SLFN11 is one example that could be inhibited for therapeutic effect. Other interactions involving distinct antiviral pathways could be targeted simultaneously to inhibit viral replication potently, for example between HCMV UL145 and HLTF. Alternatively, compounds that inhibit CRL function could be used in anti-HCMV therapy. It has been demonstrated that MLN4924 inhibits HCMV genome replication *in vitro* at nanomolar concentrations (31), but, to our knowledge, this compound has yet to be tested against HCMV in any clinical setting. Finally, our data are likely to identify further proteins that have roles in restricting infection by HCMV or other viruses.

Materials and Methods

Extended materials and methods can be found in the supplementary information (SI)

Viral infections for proteomic screens

HCMV strain Merlin was used in the PFA screen (38). Where indicated, cells were incubated with 300 μg/ml PFA (carrier: water) from the time of infection. For the block deletion mutant screen, 10 of the 11 block HCMV deletion mutants have been described previously (12). The ΔRL1-6 block deletion mutant was generated in the same fashion on the strain Merlin background lacking UL16 and UL18 and expressed a UL32-GFP reporter (wt2) (all viral recombinants used are shown in **Dataset S5A**). Detailed methods for whole cell lysate protein preparation and digestion, peptide labelling with TMT, HpRP fractionation, liquid chromatography-mass spectrometry, and data analysis are provided in the SI.

Immunoprecipitation

Cells were harvested in lysis buffer, tumbled on a rotator and then clarified by centrifugation and filtration. After incubation with immobilised mouse monoclonal anti-V5 agarose resin, samples were washed and then subjected either to immunoblotting or to mass spectrometry (see SI).

Plasmid construction and transduction

Lentiviral expression vectors encoding SLFN11, SLFN11-HA, or the V5-tagged viral proteins RL1, RL5A, RL6 and UL34 (control) were synthesised by PCR amplification and then cloned into Gateway vectors (50). V5-tagged RL1 point mutants were generated by PCR site-directed mutagenesis. For shRNA, two partially complementary oligonucleotides were annealed, and the resulting product was ligated into the pHR-SIREN vector. The primers and templates used are described in **Dataset S5C**. Stable cell lines were generated by transduction with lentiviruses produced via the transfection of HEK293T cells with the lentiviral expression vectors and helper plasmids.

siRNA knockdown

HFFF-TERTs constitutively expressing RL1-V5 or control were transfected with pools of siRNAs for CUL4A, CUL4B, a mixture of CUL4A and CUL4B, DDB1 or non-targeting siRNAs (Dharmafect) with RNAiMAX (Thermo). Cellular lysates were harvested 48 h post transfection for immunoblotting.

For infection experiments, HFFF-TERTs were transfected twice with pools of siRNA. 48 h after the first transfection, cells were passaged for re-transfection the following day and cells were infected with wt HCMV 24 h after the second transfection. Cellular lysates were harvested 72 h post infection.

Immunoblotting

Protein concentration was measured in lysed cells using a bicinchoninic acid (BCA) assay. Aliquots (50 µg) of denatured, reduced protein was separated by SDS polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, and probed using the primary and secondary antibodies detailed in SI. Fluorescent signals were detected using the Odyssey CLx Imaging System (LI-COR), and images were processed and quantified using Image Studio Lite V5.2 (LI-COR).

Plaque assay

HFFF-TERTs stably expressing shRNA constructs targeted against SLFN11 or control, or overexpressing SLFN11 or control, were infected in triplicate at MOI 0.005 with RCMV-288 (strain AD169 expressing enhanced green fluorescent protein under the control of the HCMV β-2.7 early promoter) (39). The medium was then replaced with a 1:1 (v/v) mixture of 2 x DMEM and Avicel (2% (w/v) in water). This mixture was removed 2 weeks after infection and the cells were washed then fixed in 4% (w/v) paraformaldehyde. The number of plaques per well was counted on the basis of GFP fluorescence. Plaque area was calculated using Image J Fiji software.

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Figure Legends

Figure 1. Host proteins targeted for downregulation by HCMV late during infection, identified using the viral DNA synthesis inhibitor PFA

(A) Schematic of the experimental workflow. HFFFs were infected with HCMV at multiplicity of infection (MOI) 10, and cells were harvested at the indicated times. A high multiplicity of infection was chosen in order to be consistent with our previous publications (7, 10, 11), and to infect as close as possible 100% of cells. This ensured that the ratios for protein downregulation were not compressed by proteins expressed (and not downregulated) in uninfected cells..

(B) Hierarchical cluster analysis of 527 proteins downregulated ≥3-fold by 96 hpi. For each protein, the ratios of protein expression in the presence or absence of PFA are shown. To be considered a 'hit' in the screen, proteins were additionally required to be rescued >2-fold by PFA. Enlargements to the right of the panel show examples of subclusters.

(C) Examples of temporal profiles of proteins rescued from downregulation by PFA.

Figure 2. HCMV RL1 is necessary and sufficient for downregulation of SLFN11

(A) (left panel) Numbers of human proteins targeted by each gene block using sensitive scoring (z-score >4 and FC >1.5). For each block, the z-scores of all proteins that passed scoring criteria are shown (right panel). All viruses were examined in duplicate or triplicate across three separate experiments, the first two of which we have published previously (7) (**Figure S3A**). Infection was at MOI 10 for 72 h. Further details are given in Materials and Methods and SI Methods.

(B) Table of 17 proteins that were downregulated >3-fold during HCMV infection, rescued >2-fold by PFA (**Figure 1B**), and passed sensitive scoring criteria to identify the targeting gene block.

(C) Examples of data for proteins listed in (B). In the left panels, bars of the same colour represent biological replicates (see also Figure S3A).

(D) Immunoblot confirming that RL1 alone is sufficient for downregulation of SLFN11 in stably transduced HFFF-TERTs (top panel) and transiently transfected HEK-293s (bottom panel). As we reported previously (9), expression of RL5A and RL6 was not detected by immunoblot, whereas both were detected by mass spectrometry (**Figure 3A, Dataset S4**).

(E) RL1 is necessary for downregulation of SLFN11. HFFF-TERTs were infected at MOI 10 for 72h with wt Merlin-strain HCMV, a single ΔRL1 deletion mutant and the ΔRL1-6 block deletion mutant.

(F) Expression of RL1 during HCMV infection is inhibited by PFA (left panel). The temporal profile of RL1 expression correlates inversely with expression of SLFN11 during HCMV infection (right panel). Data for each protein is shown from the 'PFA screen' proteomic experiment (**Figure 1A**). Although RL1 expression could not be directly validated due to the lack of reagents that detect its expression in the context of HCMV infection, two peptides unique only to RL1 were quantified (**Figure S4**).

Figure 3. HCMV RL1 degrades SLFN11 via the CRL4 Complex

(A) (left panel) Schematic of SILAC immunoprecipitation. HFFF-TERTs stably transduced with C-terminally V5-tagged RL1 or RL5A or RL6 as controls were treated with 10 μM MG132 for 12 h prior to harvest. (right panel) Proteins enriched >3-fold in RL1-expressing cells compared with RL6-expressing cells are shown. p-values were estimated using significance A values, then corrected for multiple hypothesis testing (21). Full data are shown in **Dataset S4**.

(B) Co-immunoprecipitation showing that RL1 interacts with DDB1. HEK-293s were stably transduced with RL1-V5 construct or controls. Input represents 1% of the sample. Proteins were detected with antibodies against V5 and DDB1.

(C) Co-immunoprecipitation showing that interaction of RL1 and DDB1 is dependent largely on residues conserved between RL1 and UL145 (right panel, conserved residues shown in blue; UL145 residues required for interaction with DDB1 in red squares (19); RL1 residues required for interaction with DDB1 in green squares). HEK-293s were stably transduced with the indicated C-terminally V5-tagged RL1 constructs. Input represents 1% of the sample. Proteins were detected with antibodies against V5 and DDB1.

(D) Immunoblot showing that SLFN11 downregulation is dependent on CUL4A, CUL4B and the adaptor protein DDB1. HFFF-TERTs stably expressing RL1-V5 or control were transfected for 48 h with siRNAs targeted against CUL4A, CUL4B, CUL4A/B, DDB1 or control.

(E) Immunoblot showing that knockdown of CULA/CUL4B and DDB1 rescues SLFN11 expression during HCMV infection. HFFF-TERTs were transfected for 48 h with siRNA targeted against CUL4A, CUL4B, DDB1 or control and then re-transfected for an additional 72 h.

(F) Inhibition of CRL activity rescues SLFN11 levels. HFFF-TERTs stably transduced with RL1-V5 or control were treated with 1 μM MLN4924 for 24 h prior to harvest.

Figure 4. SLFN11 restricts HCMV infection

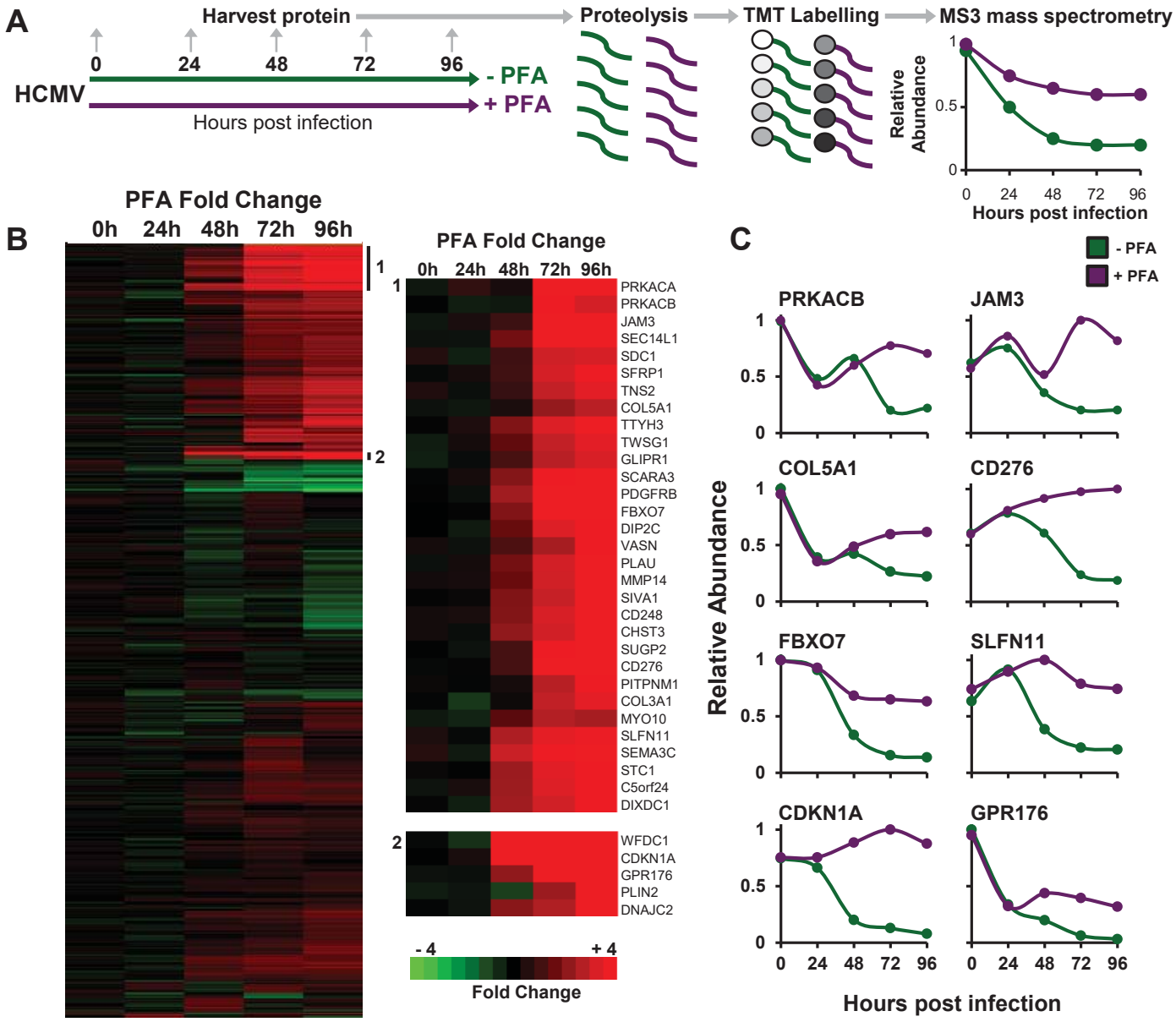
(A) SLFN11 restricts HCMV infection. HFFF-TERTs were stably transduced with shRNAs targeted against SLFN11 or control, and then infected in triplicate with AD169-GFP at MOI 0.005 under Avicel for 2 weeks before counting the number of plaques. A representative example of two experiments is shown, with error bars showing standard deviation from the mean. p-values were estimated using a two-tailed t-test (n=3). * $p<0.05$, ** $p<0.0005$. Immunoblot confirmed knockdown of SLFN11 (lower panel).

(B) SLFN11 restricts cell-cell spread of HCMV. Plaque area was calculated using Fiji software (22) using pictures of plaques from the experiment described in (A). Representative examples are shown in the right panels. p-values were estimated using a non-parametric Mann-Whitney U test (n=30). * $p<0.0005$, ** $p<0.000005$, *** $p<5x10^{-10}$.

(C) Confirmation that SLFN11 restricts HCMV infection. The experiment was conducted as described in (A), using HFFF-TERTs stably overexpressing SLFN11 or two independent control cell lines. Immunoblot confirmed overexpression of SLFN11 (lower panel).

(D-E) Multi-step growth curves confirm that SLFN11 restricts HCMV infection. HFFF-TERTs stably knocked down for SLFN11 (sh2) or control (ctrl2) were infected in duplicate with AD169-GFP at MOI 1 or 0.1 (D) or the ΔRL1-6 block deletion mutant at MOI 1 (E). Culture supernatant was harvested every 2 days and used to infect fresh HFFF-TERTs, where GFP expression at 24h (AD169-GFP) or 72h (ΔRL1-6 block deletion mutant) was used to determine viral titre (GFP^+ cells/ml of supernatant). p-values were estimated using a paired 2-way ANOVA with Tukey's HSD test for multiple comparisons (n=2). * $p<0.05$, ** $p<0.001$, *** $p<0.0001$.

Figure 1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Proteolysis
TMT Labelling
MS3 mass spectrometry
0
24
48
72
96
HCMV
- PFA
+ PFA
Hours post infection
Relative Abundance
1
0.5
0
0 24 48 72 96
Hours post infection
B
PFA Fold Change
0h 24h 48h 72h 96h
1
2
PFA Fold Change
0h 24h 48h 72h 96h
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PRKACB
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SDC1
SFRP1
TNS2
COL5A1
TTYH3
TWSG1
GLIPR1
SCARA3
PDGFRB
FBXO7
DIP2C
VASN
PLAU
MMP14
SIVA1
CD248
CHST3
SUGP2
CD276
PITPNM1
COL3A1
MYO10
SLFN11
SEMA3C
STC1
C5orf24
DIXDC1
WFDC1
CDKN1A
GPR176
PLIN2
DNAJC2
- 4
+ 4
Fold Change
C
- PFA
+ PFA
PRKACB
JAM3
COL5A1
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0
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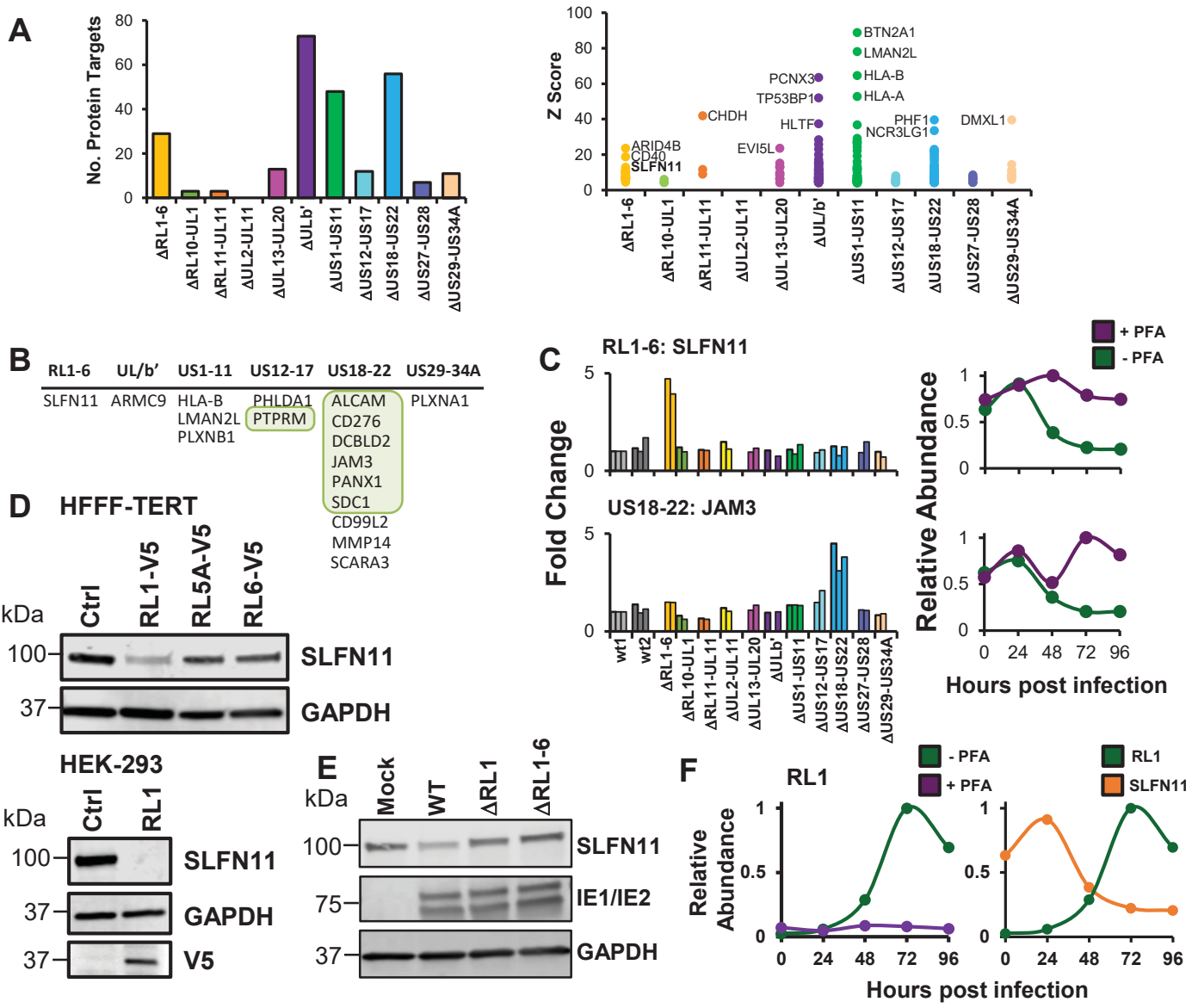
Hours post infection

Figure 2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ΔULb'
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ΔUS18-US22
ΔUS27-US28
ΔUS29-US34A
Z Score
ARID4B
CD40
SLFN11
CHDH
EVI5L
PCNX3
TP53BP1
HLTF
BTN2A1
LMAN2L
HLA-B
HLA-A
PHF1
NCR3LG1
DMXL1
B
RL1-6
UL/b'
US1-11
US12-17
US18-22
US29-34A
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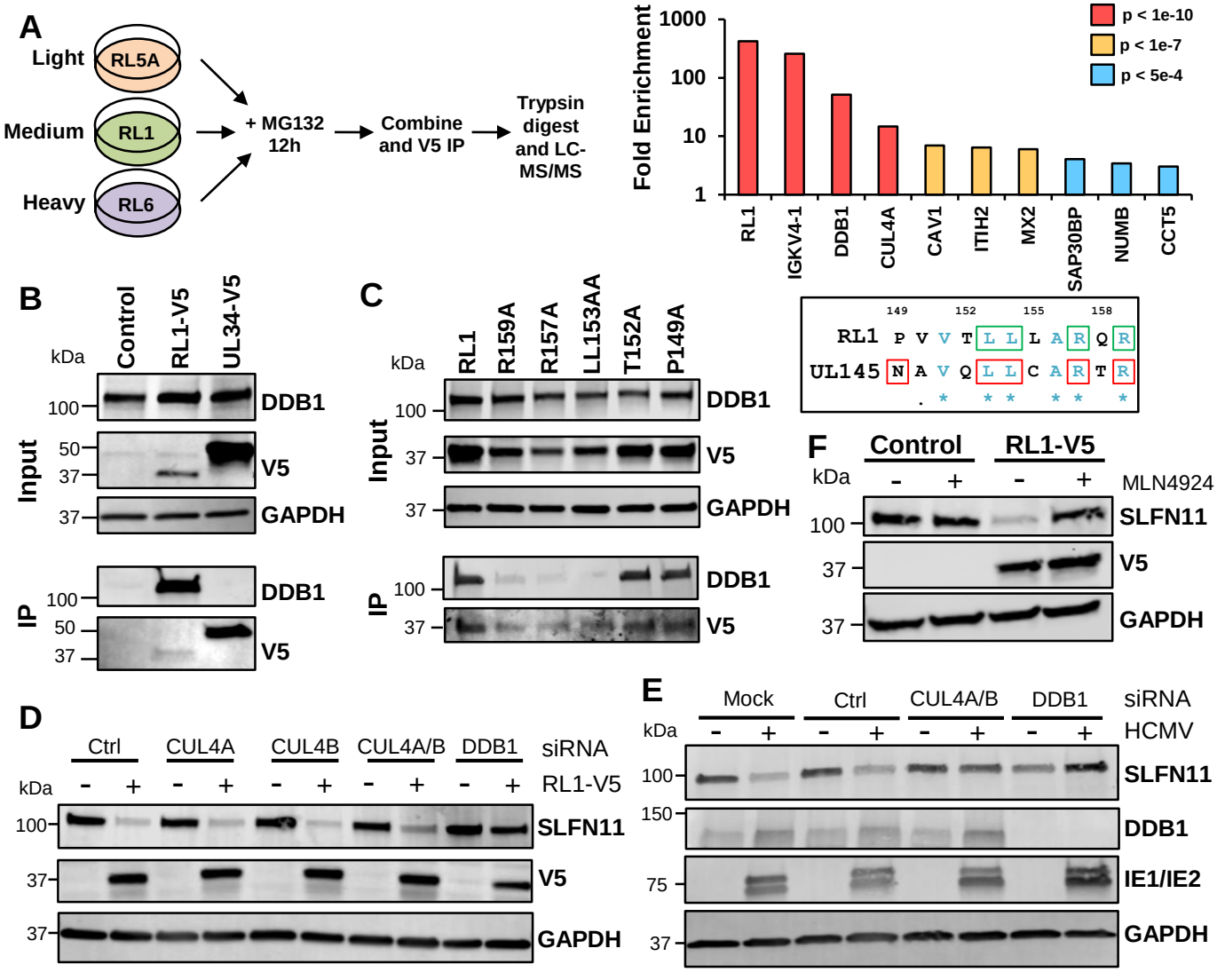
SDC1
CD99L2
MMP14
SCARA3
PLXNA1
C
RL1-6: SLFN11
US18-22: JAM3
Fold Change
wt1
wt2
+ PFA
- PFA
Relative Abundance
Hours post infection
D
HFFF-TERT
Ctrl
RL1-V5
RL5A-V5
RL6-V5
kDa
SLFN11
GAPDH
HEK-293
V5
E
Mock
WT
ΔRL1
ΔRL1-6
IE1/IE2
F
RL1
SLFN11

Figure 3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RL1
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RL6
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Fold Enrichment
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100
10
1
p < 1e-10
p < 1e-7
p < 5e-4
RL1
IGKV4-1
DDB1
CUL4A
CAV1
ITIH2
MX2
SAP30BP
NUMB
CCT5
B
Control
RL1-V5
UL34-V5
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DDB1
Input
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V5
37
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IP
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DDB1
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V5
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GAPDH
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V5
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MLN4924
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SLFN11
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V5
37
GAPDH
D
Ctrl
CUL4A
CUL4B
CUL4A/B
DDB1
siRNA
kDa
- + - + - + - + - +
RL1-V5
100
SLFN11
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V5
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GAPDH
E
Mock
Ctrl
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DDB1
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DDB1
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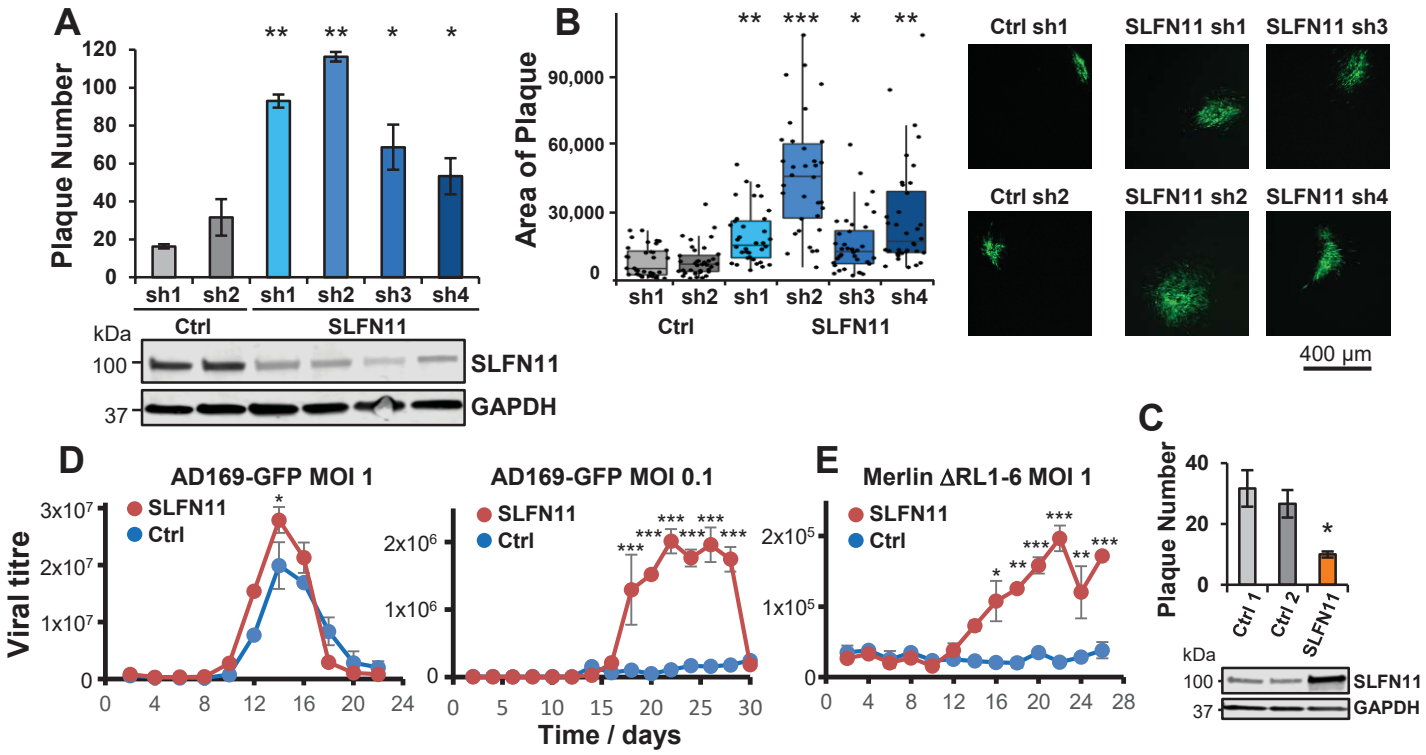
IE1/IE2
37
GAPDH

Figure 4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37
SLFN11
GAPDH
B
Area of Plaque
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Ctrl
SLFN11
Ctrl sh1
SLFN11 sh1
SLFN11 sh3
Ctrl sh2
SLFN11 sh2
SLFN11 sh4
400 µm
C
Plaque Number
Ctrl 1
Ctrl 2
SLFN11
kDa
100
37
SLFN11
GAPDH
D
AD169-GFP MOI 1
AD169-GFP MOI 0.1
SLFN11
Ctrl
Viral titre
Time / days
E
Merlin ΔRL1-6 MOI 1
SLFN11
Ctrl