

1 **The cellular NMD pathway restricts Zika virus infection and is targeted by the viral capsid**
2 **protein**

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15

16 **Abstract**

17 **Zika virus (ZIKV) infection of neural progenitor cells (NPCs) *in utero* is associated with**
18 **neurological disorders, such as microcephaly¹, but a detailed molecular understanding**
19 **of ZIKV-induced pathogenesis is lacking. Here we show that *in vitro* ZIKV infection of**
20 **human cells, including NPCs, causes disruption of the nonsense-mediated mRNA decay**
21 **(NMD) pathway. NMD is a cellular mRNA surveillance mechanism that is required for**
22 **normal brain size in mice²⁻⁴. Using affinity purification-mass spectrometry, we identified**
23 **multiple cellular NMD factors that bind to the viral capsid protein, including the central**
24 **NMD regulator up-frameshift protein 1 (UPF1)⁵. Endogenous UPF1 interacted with the**
25 **viral capsid protein in co-immunoprecipitation experiments and capsid expression post-**
26 **transcriptionally downregulated UPF1, a process that we confirmed occurs during *de***

1 **novo ZIKV infection. A further decrease in UPF1 levels by RNAi significantly enhanced**
2 **ZIKV infection in NPC cultures. We therefore propose that ZIKV, via the capsid protein,**
3 **has evolved a strategy to dampen antiviral activities of NMD^{6,7}, which subsequently**
4 **contributes to neuropathology *in vivo*.**

5

6 **Main**

7 ZIKV is a mosquito-borne RNA virus that belongs to the *Flaviviridae* family. First isolated
8 in Uganda in 1947, ZIKV remained relatively obscure for decades following its discovery
9 because infection was associated with only mild disease. However, more severe clinical
10 manifestations, including microcephaly, have been observed during the recent spread of ZIKV
11 through the Americas⁸. While it is now established that ZIKV infection during pregnancy is a
12 causative agent of microcephaly⁹, the molecular mechanisms underlying ZIKV-induced
13 neuropathogenesis remain largely unknown.

14 Microcephaly has been linked to genetic mutations that result in the impairment of the
15 NMD pathway²⁻⁴. While NMD was initially found to serve as a quality control system that
16 destroys transcripts containing premature termination codons, the pathway also targets a
17 broader range of RNA substrates, including viral RNAs^{5-7,10}. As ZIKV has an RNA genome, and
18 we previously described perturbations of the NMD pathway in cells infected with hepatitis C
19 virus¹¹, we hypothesized that ZIKV infection manipulates the cellular NMD pathway.

20 To determine if ZIKV infection affects NMD, we infected Huh7 human hepatic cells and
21 human induced pluripotent stem cell (iPSC)-derived NPCs with ZIKV for 48 h. We isolated total
22 RNA from infected cells and measured mRNA levels of three canonical NMD substrates:
23 asparagine synthetase (ASNS), cysteinyl-tRNA synthetase (CARS), and SR protein SC35¹¹.
24 ASNS, CARS, and SC35 transcripts were significantly elevated in Huh7 cells and NPCs
25 following infection with Asian lineage ZIKV strain P6-740 (Fig. 1a). Levels of NMD substrates
26 were also elevated in Huh7 cells infected with the contemporary ZIKV clinical isolate

1 PRVABC59 (Puerto Rico, 2015)(Fig. 1a). We found that ZIKV-induced increase in NMD
2 transcripts did not reflect a global increase in transcription, as mRNA levels of housekeeping
3 genes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were not altered in
4 infected cells (Fig. 1a). Together, these results indicate that ZIKV disrupts the NMD pathway
5 during infection.

6 NMD substrates are regulated through the activity of UPF1, an evolutionarily conserved
7 ATP-dependent RNA helicase. UPF1 plays a central role in the NMD pathway by linking the
8 translation termination event to the assembly of a surveillance complex, resulting in NMD
9 activation¹². To determine if ZIKV infection more broadly affects NMD, we utilized two publicly
10 available RNA-Seq datasets to compare genome-wide transcriptional alterations found during
11 ZIKV infection¹³ to those found following UPF1 knockdown¹⁴. As shown in Figure 1b, there is a
12 significant overlap in upregulated genes between these two datasets. Interestingly, several of
13 the overlapping genes are involved in cell cycle arrest and induction of apoptosis, two conditions
14 linked to ZIKV-associated neuropathology¹. These genes include DNA damage-inducible
15 transcript 3 (DDIT3)¹⁵ and growth arrest and DNA damage-inducible protein 45 alpha and beta
16 (GADD45A and GADD45B, respectively)¹⁶. Via quantitative real-time RT-PCR, we confirmed
17 that transcripts of each were upregulated following infection of Huh7 cells with ZIKV
18 PRVABC59, while the mRNA levels of the housekeeping genes GAPDH, hypoxanthine
19 phosphoribosyltransferase 1 (HPRT1), and lactate dehydrogenase A (LDHA) were not elevated
20 (Fig. 1c). Combined, these data show that ZIKV infection is associated with dysregulated
21 expression of NMD substrates relevant to ZIKV-mediated neuropathogenesis.

22 We previously showed that the core protein of HCV and capsid protein of the related
23 flaviviruses dengue virus and West Nile virus interact with within bgn homolog (WIBG/PYM1),
24 a component of the exon junction complex (EJC) associated with NMD¹¹. To examine potential
25 interactions between ZIKV capsid and NMD-associated host factors, we used an affinity
26 purification with mass spectrometry (AP-MS) approach to generate ZIKV capsid-host protein-

1 protein interaction (PPI) maps in HEK293T cells; ZIKV capsid from the Ugandan 1947 strain MR
2 766 or ZIKV capsid from the French Polynesian 2013 strain H/PF/2013 served as bait proteins
3 (Shah et al., submitted). Three independent experiments were performed for each tagged ZIKV
4 capsid bait protein, with tagged GFP and empty vector transfections used as negative controls.
5 From these studies, we found that ZIKV capsid proteins interacted with several factors of the
6 NMD pathway, including multiple members of the EJC complex, as well as UPF1 and UPF3B
7 (UPF3B is an NMD effector that stimulates the helicase activity of UPF1) (Fig. 2a). Importantly,
8 the NMD host factors that interact with each of the two different capsid proteins greatly
9 overlapped, revealing that the interaction between capsid and the NMD pathway is conserved
10 across the Asian and African lineages of ZIKV (Fig. 2a).

11 Next, we validated the binding of ZIKV capsid to UPF proteins by co-
12 immunoprecipitating Flag-tagged capsid protein with endogenous UPF3B or UPF1 in HEK293T
13 cells. Both UPF3B and UPF1 proteins co-immunoprecipitated with ZIKV capsid, confirming the
14 AP-MS results (Fig. 2b,c, respectively). Surprisingly, we consistently observed a decrease in
15 UPF1, but not UPF3B, protein levels in the input lysate of ZIKV capsid-transfected cells,
16 pointing to a specific perturbation of UPF1 expression by ZIKV capsid (Fig. 2c). Because ZIKV
17 capsid and UPF1 both localize to the nucleus and the cytoplasm^{17,18}, we also performed cellular
18 fractionation studies in ZIKV capsid-transfected HEK293T cells. Capsid expression markedly
19 decreased nuclear UPF1 levels, whereas cytoplasmic levels were unchanged (Fig. 2d). As
20 UPF1 transcript levels are not altered in ZIKV-infected NPCs¹³, and changes in UPF1
21 transcripts cannot explain the compartment-specific decrease in protein levels, we focused on
22 mechanisms known to mediate nuclear degradation of proteins. Interestingly, nuclear UPF1
23 levels in capsid-transfected cells were not rescued by inhibition of either the ubiquitin-
24 proteasome pathway via MG132 treatment or cellular autophagy via spautin-1 and bafilomycin
25 A1 treatment, indicating that the mechanism of ZIKV capsid-mediated UPF1 protein
26 downregulation is uncommon (Supplemental Fig. 1a,b, respectively). These data identified a

1 new interaction between ZIKV capsid and the NMD pathway that perturbs nuclear UPF1 levels
2 through a yet-unknown mechanism. Notably, we detected no double-stranded viral RNA
3 (dsRNA) in the nuclei of ZIKV-infected NPCs (Supplemental Fig. 2a,b and Supplemental Video
4 1), despite reports that flavivirus RNA or RNA replication is localized to the nucleus¹⁹. This
5 suggested that UPF1 is not targeted by ZIKV capsid to protect nuclear viral RNA from
6 degradation.

7 To confirm that UPF1 protein levels are dysregulated during *de novo* ZIKV infection, we
8 performed western blot analysis of infected Huh7 cells and NPCs. Cellular UPF1 protein levels
9 were consistently downregulated by ~50% in ZIKV-infected Huh7 cells, whereas a ~25%
10 reduction was observed in ZIKV-infected NPCs (Fig. 3a,b, respectively). This difference in UPF1
11 downregulation mirrors the difference in infection efficiencies achieved in these two cell
12 systems. As expected, UPF1 transcript levels were not lower in ZIKV-infected cells than in their
13 mock-infected counterparts (Fig. 3c). In addition, no specific effect was observed on UPF1
14 phosphorylation, a mechanism known to activate UPF1⁵, as the decrease detected in
15 phosphorylated UPF1 levels corresponded with the reduction in total UPF1 levels in ZIKV-
16 infected cells (Fig. 3d). These results confirm that UPF1 is post-transcriptionally downregulated
17 during ZIKV infection.

18 We hypothesized that UPF1 serves as a restriction factor of ZIKV and is inactivated in
19 infected cells to promote ZIKV propagation. To test this hypothesis, we decreased UPF1
20 expression prior to ZIKV infection by transfecting NPCs with either non-targeting siRNA or a
21 pool of UPF1-specific siRNAs. We then infected the transfected cells with ZIKV and measured
22 viral RNA levels, as well as infectious titers, 48 h post-infection (hpi). UPF1 knockdown was
23 successful in siRNA-treated cells, as confirmed by western blot analysis (Fig. 4a). The depletion
24 of UPF1 in NPCs prior to infection resulted in a significant increase in both ZIKV RNA replication
25 and infectious virus production (Fig. 4b,c respectively), supporting the model that expression of
26 UPF1 restricts ZIKV infection. Using confocal microscopy and 3D reconstruction analyses, we

1 observed no significant difference in the number and size of dsRNA foci when we compared
2 ZIKV-infected, UPF1-depleted NPCs to ZIKV-infected cells expressing UPF1 (Fig. 4d). Instead,
3 we found a consistent increase in the number of infected cells in NPC cultures when UPF1 was
4 depleted (Fig. 4e), although this increase was not significant due to the variability in infection
5 efficiencies across the two different NPC lines used. Combined, these data suggest that UPF1
6 expression renders NPCs more resistant to ZIKV infection, but does not target replicating viral
7 RNA.

8 In summary, we identified the NMD pathway as a restriction mechanism for ZIKV
9 infection in human NPCs. NMD was partially inactivated in ZIKV-infected NPCs through
10 expression of the viral capsid protein and the resulting downregulation of host UPF1 protein
11 levels. As weakening NMD by depleting UPF1 results in a marked increase in infection
12 efficiency and favors successful ZIKV spread, we propose a model in which an “arms race”
13 between cellular NMD and ZIKV determines whether a cell is successfully infected. The
14 downregulation of UPF1 by capsid during ZIKV infection may be limited by potentially toxic
15 effects of NMD impairment, as illustrated by the upregulation of genes regulating cell cycle
16 growth arrest and apoptosis. Indeed, ZIKV-induced NMD impairment may contribute to severe
17 neuropathology and microcephaly development, as documented in mice haploinsufficient for
18 NMD factors²⁻⁴. Research is ongoing to determine the precise molecular mechanisms of ZIKV
19 capsid-induced UPF1 downregulation and the specific role of nuclear UPF1 in ZIKV infection.
20 This research may lead to new therapeutic approaches, as reinforcement of the antiviral
21 properties of the cellular NMD pathway is expected to enhance resistance of NPCs to ZIKV
22 infection and could promote normal neurodevelopment in infected fetuses.

23

24 **Methods**

25 **Viruses and cells.** Two Asian lineage strains of ZIKV, P6-740 (ATCC VR-1845) and
26 PRVABC59 (ATCC VR-1843), were used for all experiments. ZIKV stocks were propagated in

1 Vero cells (ATCC) and titers were determined by plaque assays on Vero cells. Huh7 cells
2 (ATCC) and Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with
3 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL
4 streptomycin. HEK293T cells (ATCC) were maintained in DMEM/H21 medium supplemented
5 with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 mM sodium pyruvate or
6 DMEM with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.
7 Human iPSC-derived NPCs were generated and maintained as described previously²¹. All of the
8 human fibroblast cell lines used to generate iPSCs came from the Coriell Institute for Medical
9 Research. The iPSCs used in these studies were the CTRL2493nXX, CS2518nXX, and
10 Cs71iCTR-20nXX lines. CTRL2493nXX was derived from the parental fibroblast
11 line ND31845 that was biopsied from a healthy female at 71 years of age. The iPSC line was
12 made by the Yale Stem Cell Center. CS2518nXX was derived from the parental fibroblast line
13 ND30625 that was biopsied from a healthy male at 76 years of age. The iPSC line comes from
14 the Coriell Institute for Medical Research. CS71iCTR-20nXX was derived from the parental
15 fibroblast line ND29971 that was biopsied from a female at 61 years of age. This iPSC line
16 comes from the Coriell Institute for Medical Research. For virus infections, NPCs plated on
17 Matrigel-coated multi-well plates or Huh7 cells were infected with ZIKV at a multiplicity of
18 infection of 0.1 or 1 for 2 h at 37°C. Infected cells were harvested at 48 hpi for all analyses.

19
20 **Affinity purification, mass spectrometry, and AP-MS scoring.** The ZIKV capsid open
21 reading frames (ORFs) from the Ugandan 1947 strain MR 766 or the French Polynesian 2013
22 strain H/PF/2013 were cloned into pCDNA4_TO with a C-terminal 2xStrep II affinity tag for
23 expression in human cells. The viral capsid proteins (three biological replicates), as well as GFP
24 (two biological replicates) and empty vector (ten biological replicates) as negative controls, were
25 expressed in HEK293T cells and affinity purifications were performed as previously described²¹.
26 All lysates and affinity purified eluates were analyzed by western blot and silver stain PAGE to

1 confirm expression and purification. Purified protein eluates were digested with trypsin for LC-
2 MS/MS analysis. Samples were denatured and reduced in 2M urea, 10 mM NH₄HCO₃, 2 mM
3 DTT for 30 min at 60°C, then alkylated with 2 mM iodoacetamide for 45 min at room
4 temperature. Trypsin (Promega) was added at a 1:100 enzyme:substrate ratio and digested
5 overnight at 37°C. Following digestion, samples were concentrated using C18 ZipTips
6 (Millipore) according to the manufacturer's specifications. Peptides were resuspended in 15 µL
7 of 4% formic acid and 3% ACN, and 1-2 µL of sample was loaded onto a 75 µm ID column
8 packed with 25 cm of Reprosil C18 1.9 µm, 120Å particles (Dr. Maisch). Peptides were eluted
9 into a Q-Exactive Plus (Thermo Fisher) mass spectrometer by gradient elution delivered by an
10 Easy1200 nLC system (Thermo Fisher). The gradient was from 4.5% to 32% acetonitrile over
11 53 minutes. All MS spectra were collected with orbitrap detection, while the 20 most abundant
12 ions were fragmented by HCD and detected in the orbitrap. All data was searched against the
13 SwissProt Human protein sequences, combined with ZIKV sequences and GFP. Peptide and
14 protein identification searches, as well as label-free quantitation, were performed using the
15 MaxQuant data analysis algorithm and all peptide and protein identifications were filtered to a
16 1% false-discovery rate^{22,23}. SAINTq (PMID: 27119218) was used to calculate the probability of
17 bait-prey interactions for both Ugandan ZIKV capsid and French Polynesian ZIKV capsid
18 against the negative controls, including GFP and empty vector, with protein intensities as input
19 values. We applied a combined threshold of probability of interaction (AvgP) greater than 0.90
20 and Bayesian False Discovery Rate of less than 0.05.

21

22 **Quantitative real-time reverse transcription-PCR (qRT-PCR).** Total cellular RNA was
23 isolated from Huh7 cells and NPCs using the RNeasy Mini Kit (Qiagen). cDNA was synthesized
24 with oligo(dT)₁₈ (ThermoFisher Scientific) primers, random hexamer (Life Technologies)
25 primers, and AMV reverse transcriptase (Promega). The cDNA was then used in SYBR Green

1 PCR Master Mix (ThermoFisher Scientific) according to manufacturer's instructions and
2 analyzed by qPCR (Bio-Rad ABI 7900). The primers used for ASNS, CARS, SC35 1.7, GAPDH,
3 HPRT1, LDHA, and 18S rRNA have been described previously¹¹. The additional primers used
4 were ZIKV PRVABC59 forward primer 5'- GAG ACG AGA TGC GGT ACA GG -3', ZIKV
5 PRVABC59 reverse primer 5'- CGA CCG TCA GTT GAA CTC CA -3', UPF1 forward primer 5'-
6 CTG CAA CGG ACG TGG AAA TAC -3', UPF1 reverse primer 5'- ACA GCC GCA GTT GTA
7 GCA C -3', DDIT3 forward primer 5'- CTG CTT CTC TGG CTT GGC TG -3', DDIT3 reverse
8 primer 5'- GCT CTG GGA GGT GCT TGT GA -3', GADD45A forward primer 5'- GAG CTC CTG
9 CTC TTG GAG AC -3', GADD45A reverse primer 5'- GCA GGA TCC TTC CAT TGA GA -3',
10 GADD45B forward primer 5'- TGA CAA CGA CAT CAA CAT C -3', and GADD45B reverse
11 primer 5'- GTG ACC AGA GAC AAT GCA G -3'. Relative levels of each transcript were
12 normalized by the delta threshold cycle method to the abundance of 18S rRNA, with mock-
13 infected cells set to 1.

14

15 **Western blot analysis.** Cells were lysed in RIPA lysis buffer (50mM Tris-HCl, pH 8, 150mM
16 NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with Halt™ protease
17 inhibitor cocktail (ThermoFisher Scientific) to obtain whole cell lysates or lysed using the NE-
18 PER nuclear and cytoplasmic extraction kit (ThermoFisher Scientific) to obtain cytoplasmic and
19 nuclear fractions. Proteins were separated by SDS-PAGE and transferred to nitrocellulose
20 membranes (Bio-Rad). Blots were incubated with the indicated primary antibody: anti-phospho-
21 UPF1 (Ser1127) (07-1016, Millipore Sigma), anti-UPF1 (12040, Cell Signaling Technology,
22 Inc.), anti-UPF3B (ab134566, Abcam), anti-ZIKV Envelope (E) (GTX133314, GeneTex), anti-
23 ZIKV Capsid (C) (GTX133304, GeneTex), anti-Flag (F7425, Sigma-Aldrich), anti-SP1 (sc14027,
24 Santa Cruz Biotechnology), anti-GAPDH (5174, Cell Signaling Technology, Inc.), anti-β-actin
25 (A5316, Sigma-Aldrich). Proteins were visualized by chemiluminescent detection with ECL and

1 ECL Hyperfilm (Amersham). Differences in band intensity were quantified by densitometry using
2 ImageJ.

3

4 **Immunoprecipitations.** Cells were lysed in either RIPA lysis buffer or IP lysis buffer (150mM
5 NaCl, 50mM Tris pH 7.4, 1mM EDTA, 0.5% NP-40 substitute, supplemented with Halt™
6 protease inhibitor cocktail (ThermoFisher Scientific)) at 4°C and passed through a G23 needle.
7 Clarified lysates were immunoprecipitated with Flag M2 agarose (Sigma) overnight, washed in
8 lysis buffer, and resuspended in Laemmli buffer for SDS-PAGE. Western blot analysis of
9 immunoprecipitated proteins was performed as described above.

10 **Immunofluorescence.** Transfected or infected NPCs were collected at 48 h and plated onto 22
11 × 22 mm #1.5 coverslips. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1%
12 Triton X-100, and blocked in 3% bovine serum albumin. Cells were then immunostained with
13 human anti-DENV mAb 1.6D (a generous gift from Sharon Isern and Scott Michael, Florida Gulf
14 Coast University), which recognizes the ZIKV envelope protein or with the anti-dsRNA mAb J2
15 (SCICONS), and the appropriate secondary antibody. Coverslips were mounted onto glass
16 slides using Vectashield® Mounting Medium with DAPI (Vector Laboratories) and analyzed by
17 fluorescence microscopy (Zeiss Axio Observer ZI) or confocal microscopy (Zeiss LSM 880). For
18 acquiring high-resolution images, cells were imaged on the Zeiss LSM 880 with Airyscan using
19 a 20x/0.8 or 63x/1.4 M27 oil immersion objective. A total of 15-20 (20x objective) or 60-80 (63x
20 objective) Z-slices were acquired every 0.88 µm or 0.3 µm, respectively. The resulting Z-stack
21 was reconstructed and rendered in three dimensions using Imaris software (Bitplane). The
22 Imaris co-localization function was used to determine overlap of DAPI and dsRNA fluorescence.
23 dsRNA foci were reconstructed via the Imaris spot detection function, which provided an
24 analysis of total number and mean volume of foci within a cell.

25

1 **Statistical analysis.** Statistical differences between groups were analyzed using either a two-
2 tailed unpaired Student's *t*-test or a two-tailed ratio paired Student's *t*-test, as stated in the figure
3 legends. Hypergeometrical tests were used to calculate the probability of an overlap in gene
4 dysregulation between ZIKV-infected NPCs and UPF1-depleted cells and to calculate the
5 probability of ZIKV capsid bait-prey interactions. Data are represented as mean \pm s.e.m.
6 Statistical significance was defined as $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, and $****P \leq 0.0001$.

7

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18

19 **Competing interests.** The authors declare no competing financial interests.

20

21 **Author contributions.** K.A.F., K.E.L., M.M.K., and P.S.S. designed, conducted, and analyzed
22 the experiments. D.J. performed the bioinformatics analyses and provided graphical support.
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24 reagents and technical guidance. The manuscript was written by K.A.F., K.E.L., and M.O. with
25 input from N.J.K.

1 **Figure Legends**

2 **Figure 1. The NMD pathway is disrupted during ZIKV infection.**

3 (a) Transcript levels of NMD substrates and housekeeping genes from Huh7 cells or NPCs
4 mock-infected or infected with ZIKV strain P6-740 or the contemporary clinical isolate
5 PRVABC59. Cells were infected at a multiplicity of infection (MOI) of 0.1 or 1 and harvested at
6 48 hours post-infection (hpi). Data are represented as mean \pm s.e.m. *P* values were calculated
7 by unpaired Student's *t*-test. **P* \leq 0.05; ***P* \leq 0.01; ns, not significant. n= 3 independent
8 experiments.

9 (b) Venn diagram showing overlap of significantly upregulated genes associated with ZIKV
10 infection of NPCs and UPF1 knockdown in HeLa cells. RNA-Seq analyses of mock-infected or
11 ZIKV-infected NPCs harvested at 56 hpi and control siRNA-treated or UPF1 siRNA-treated
12 HeLa TO cells harvested at 72 hours post-transfection (hpt). The GeneProf hypergeometric
13 probability calculator (<http://www.geneprof.org/GeneProf/tools/hypergeometric.jsp>) was then
14 used to generate a hypergeometric *P* value. *****P* \leq 0.0001.

15 (c) Transcript levels of housekeeping genes and select genes involved in cell cycle growth
16 arrest and apoptosis that were identified in (b). Huh7 cells were mock-infected or infected with
17 ZIKV PRVABC59 at an MOI of 0.1 or 1 and harvested at 48 hpi. Data are represented as mean
18 \pm s.e.m. *P* values were calculated by unpaired Student's *t*-test. **P* \leq 0.05; ***P* \leq 0.01; ****P* \leq
19 0.001; ns, not significant. n= 3 independent experiments.

20

21 **Figure 2. The capsid protein of ZIKV interacts with the NMD pathway and downregulates**
22 **UPF1.**

23 (a) Ugandan ZIKV capsid (Ug Cap, MR 766) and French Polynesian ZIKV capsid (Fp Cap,
24 H/PF/2013) PPI maps that show significant enrichment for host NMD-associated factors
25 (purple), as identified by AP-MS (SAINTq probability score $>$ 0.9 and FDR $<$ 0.05). Ten
26 interactions between Fp Cap and host NMD factors (hypergeometrical test, *P* value = $7.16 \times 10^{-}$

1 ¹⁰) and eight interactions between Ug Cap and host NMD factors (P value = 3.45×10^{-7}) were
2 identified.

3 (b) Flag-tag co-immunoprecipitation (co-IP) and western blot analysis of HEK293T cells
4 transfected with vector or Flag-tagged ZIKV capsid (H/PF/2013, Asian lineage) and harvested at
5 48 hpt to immunoprecipitate endogenous UPF3B. The upper band detected in the IP Capsid
6 blot represents a non-specific artifact.

7 (c) Co-IP and western blot analysis of HEK293T cells transfected with vector or Flag-tagged
8 ZIKV capsid (H/PF/2013, Asian lineage) and harvested at 48 hpt to immunoprecipitate
9 endogenous UPF1.

10 (d) Western blot analysis of UPF1 levels in subcellular fractionated HEK293T cells transfected
11 with vector or Flag-tagged ZIKV capsid (H/PF/2013, Asian lineage) for 48 h. GAPDH was used
12 as a cytoplasmic marker and SP1 as a nuclear marker to ensure optimal fractionation.
13 Densitometric analyses were performed using ImageJ to quantify relative band intensities. Data
14 are represented as mean \pm s.e.m. P values were calculated by unpaired Student's t -test. $**P \leq$
15 0.01; ns, not significant. $n = 3$ independent experiments.

16

17 **Figure 3. UPF1 is post-transcriptionally downregulated during ZIKV infection.**

18 (a) Western blot analysis of UPF1 levels in mock-infected and ZIKV-infected (PRVABC59, MOI
19 of 1) Huh7 cells harvested at 48 hpi, with β -actin and ZIKV envelope protein (ZIKV E) serving as
20 loading and infection controls, respectively. Densitometric analyses were performed using
21 ImageJ to quantify relative band intensities. Data are represented as mean \pm s.e.m. P values
22 were calculated by unpaired Student's t -test. $**P \leq 0.01$. $n = 3$ independent experiments.

23 (b) Western blot analysis of UPF1 levels in mock-infected and ZIKV-infected (P6-740, MOI of 1)
24 NPCs harvested at 48 hpi, with β -actin and ZIKV capsid protein (ZIKV C) serving as loading and
25 infection controls, respectively. Densitometric analyses were performed using ImageJ to
26 quantify relative band intensities. Data are represented as mean \pm s.e.m. P values were

1 calculated by unpaired Student's *t*-test. ****P* ≤ 0.001. *n*= 3 independent experiments using one
2 NPC line.

3 (c) UPF1 transcript levels from Huh7 cells mock-infected or infected with ZIKV strain
4 PRVABC59 at an MOI of 0.1 or 1 and harvested at 48 hpi. Data are represented as mean ±
5 s.e.m. *P* values were calculated by unpaired Student's *t*-test. ns, not significant. *n*= 3
6 independent experiments.

7 (d) Western blot analysis of phosphorylated (p-UPF1) and total levels of UPF1 in mock-infected
8 and ZIKV-infected (PRVABC59, MOI of 1) Huh7 cells harvested at 48 hpi, with β-actin and ZIKV
9 E serving as loading and infection controls, respectively.

10

11 **Figure 4. UPF1 is a restriction factor of ZIKV.**

12 (a) Western blot analysis of UPF1 levels in NPCs transfected with non-targeting siRNA (siNT) or
13 a pool of UPF1-specific siRNAs (siUPF1) at 96 hpt. Densitometric analyses were performed
14 using ImageJ to quantify relative band intensities. Data are represented as mean ± s.e.m. *P*
15 value was calculated by unpaired Student's *t*-test. ***P* ≤ 0.01. *n*= 3 independent experiments
16 using one NPC line.

17 (b) ZIKV RNA levels in siNT-treated or siUPF1-treated NPCs infected with ZIKV strain
18 PRVABC59 at an MOI of 0.1 or 1 and harvested at 48 hpi. Data are represented as mean ±
19 s.e.m. *P* value was calculated by two-tailed ratio paired Student's *t*-test. ****P* ≤ 0.001.

20 (c) Released infectious virus from siNT-treated or siUPF1-treated, ZIKV-infected (PRVABC59,
21 MOI of 1) NPCs harvested at 48 hpi. Data are represented as mean ± s.e.m. *P* value was
22 calculated by unpaired Student's *t*-test. ***P* ≤ 0.01. *n*= 3 independent experiments using one
23 NPC line.

24 (d) Representative confocal microscopy images of a ZIKV-infected, siNT-treated NPC or a
25 ZIKV-infected, siUPF1-treated NPC with the nuclei stained with DAPI and ZIKV dsRNA foci
26 stained with the anti-dsRNA mAb J2. Three-dimensional image rendering and reconstructed

1 dsRNA foci were produced using the Imaris spot detection function. Measurements of dsRNA
2 foci count and volume were averaged for each cell. Data are represented as mean \pm s.e.m. n =
3 3 independent experiments using two NPC lines. Scale bar represents 2 μ m.

4 (e) Infection rates of siNT-treated or siUPF1-treated, ZIKV-infected (PRVABC59, MOI of 1)
5 NPCs measured at 48 hpi. Fixed cells were subjected to the anti-DENV mAb 1.6D, which also
6 recognizes the ZIKV envelope protein. Data are represented as mean \pm s.e.m. n = 3
7 independent experiments using two NPC lines.

8

9 **Supplemental Figure 1. ZIKV capsid-induced nuclear UPF1 downregulation occurs via a**
10 **proteasome- and autophagy-independent mechanism.**

11 (a) Western blot analysis of nuclear UPF1 levels in fractionated HEK293T cells transfected with
12 vector or Flag-tagged ZIKV capsid for 48 h. Cells were treated with DMSO or the proteasome
13 inhibitor MG132 (20 μ M) for 4 h before harvest.

14 (b) Western blot analysis of nuclear UPF1 levels in fractionated HEK293T cells transfected with
15 vector or Flag-tagged ZIKV capsid for 48 h. Cells were treated with DMSO or the autophagy
16 inhibitors spautin-1 (SP-1) (10 μ M) or bafilomycin A1 (BAF) (10 nM) for 24 h before harvest.

17

18 **Supplemental Figure 2. ZIKV RNA is not localized to the nucleus during infection.**

19 (a) Representative confocal microscopy images of a ZIKV-infected NPC (PRVABC59, MOI of 1)
20 showing antibody-stained dsRNA fluorescence and dsRNA foci as reconstructed by the Imaris
21 spot detection function. The overlay of the two images shows concordance of the dsRNA signal.
22 Scale bar represents 2 μ m.

23 (b) Representative confocal microscopy images of a mock-infected NPC and ZIKV-infected
24 (PRVABC59, MOI of 1) NPC. The pink dsRNA foci denote overlap of dsRNA fluorescence and
25 DAPI fluorescence. Scale bar represents 3 μ m.

26

1 **Supplemental Video 1. ZIKV RNA is not localized to the nucleus during infection.**

2 Representative three-dimensional video of rendered confocal Z-stacks of a ZIKV-infected NPC
3 (PRVABC59, MOI of 1), with dsRNA fluorescence reconstructed as foci by the Imaris spot
4 detection function. Scale bar represents 2 μ m.

5

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Figure 1

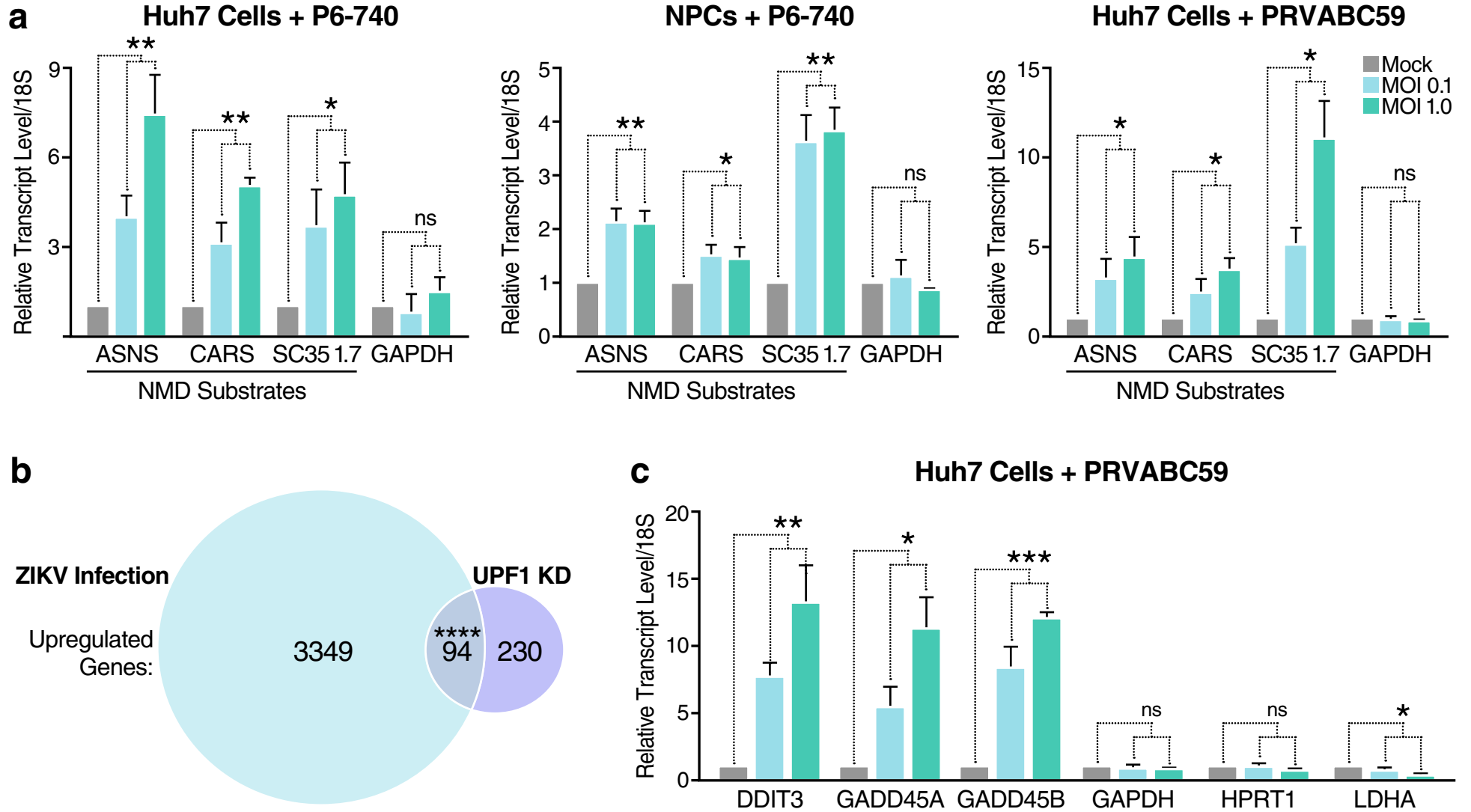


Figure 2

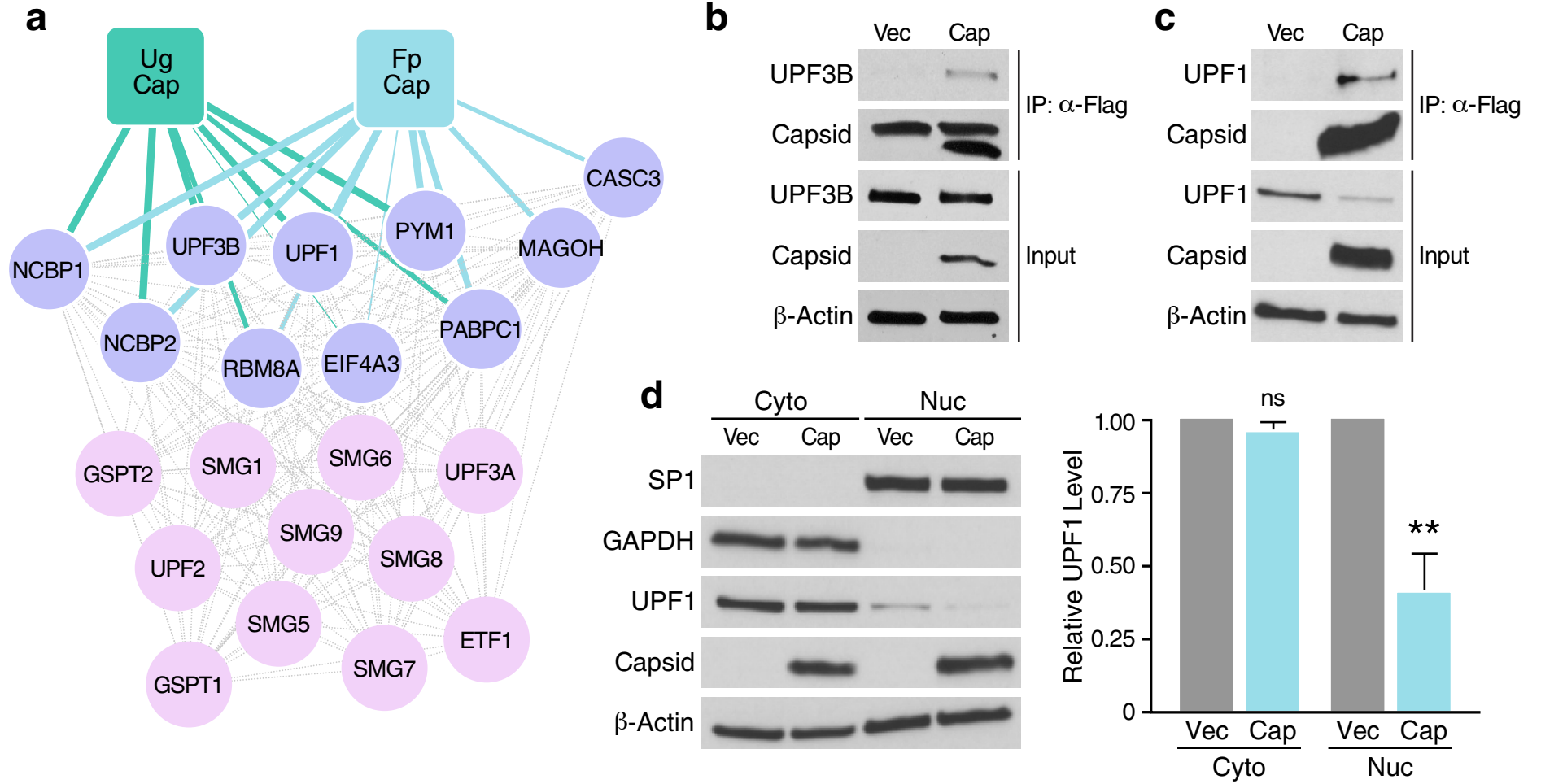


Figure 3

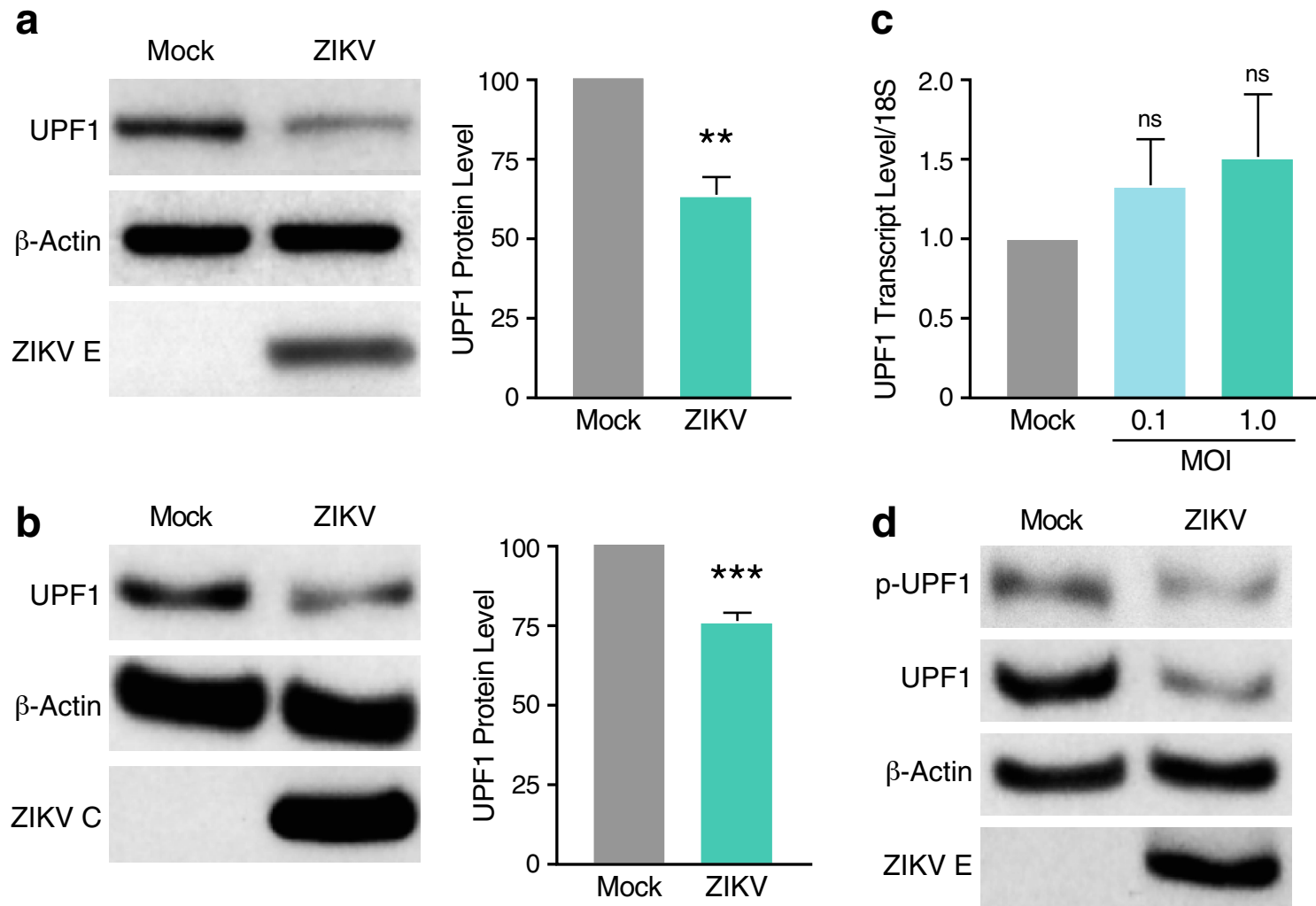


Figure 4

