Disruption of cellular proteostasis by H1N1 influenza A virus causes α-synuclein aggregation

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Supplementary Material

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Supplementary methods

Influenza A/WS/33 viral stock preparation and viral replication. The A/WS/33 H1N1 influenza strain with 1 x 10^8 PFU/µL, purchased from ATCC (ATCC VR-1520), was used to prepare the viral stocks (1). Viral propagation was performed in MDCK.2 canine cells for 5 days, in serum free DMEM medium. After the incubation time cells were harvested and centrifuged at 1000 x *g* for 5 min and the supernatant was collected and snap-frozen. Viral infectious titers were determined using the $TCID_{50}$ method (2). Briefly, MDCK.2 cells were seeded in a 96 well plate with a density of 3 x 10^4 cells per well and incubated in serum free medium with serial log_{10} viral dilutions. 72h later, the number of infected wells was counted and the tissue culture infective dose was calculated. For animal inoculations a mouse-neuroadapted influenza A virus (A/WSN/33, 1.4 x 10^5 PFU/mL; kindly provided by Dr. S. Nakajima, The Institute of Public Health, Tokyo, Japan) was used.

Culture and differentiation of LUHMES cells. Lund human mesencephalic (LUHMES) cells were purchased from ATCC. LUHMES cells were maintained in a proliferative state in DMEM:F12 medium supplemented with 1% N2 (ThermoFisher Scientific), 1% (v/v) penicillin/streptomycin (PenStrep) (Invitrogen) and 40 ng/mL bFGF (basic recombinant human Fibroblast Growth Factor) (Sigma) in a pre-coated flask with 50 µg/mL poly-L-ornithine (Sigma) and then with 1 µg/mL of laminin (Sigma). Proliferating LUHMES cells were converted into uniformly post-mitotic neurons by using a differentiation procedure, triggered by suppression of v-myc expression, as previously described (3). The differentiation procedure of LUHMES cells involves the cell maintenance in DMEM:F12 medium supplemented with 1 µg/mL tetracycline (Sigma), 2 ng/mL GDNF (glial cell line-derived neurotrophic factor) (Sigma), and 1 mM dibutyryl-cAMP (Sigma) for 5 days. The dopaminergic neuronal phenotype of differentiated LUHMES cells was described previously (3, 4).

Human neuroblastoma and MDCK.2 cells. The human neuroblastoma NLF cells were obtained mycoplasma-free from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and regularly checked for mycoplasma. 2,5 x 10^4 NLF cells were seeded onto 30 mm glass coverslips. 24h later, cells were incubated with the DNA/ metafectene complexes (METAFECTENE® PRO; Biontex) in Opti-MEM without serum (Invitrogen) for 4h, and then the transfection medium was replaced with RPMI medium (Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), 1% (v/v) L-glutamine (Invitrogen), and 1% (v/v) PenStrep for 48h. In order to assess the promoter activity of α-synuclein and DISC1 we used a pGL3 luciferase reporter vector with the canonical transcriptional start site

of the SNCA gene, and pGL4.10 with a medium DISC1 promoter sequence. These constructs were kind gifts by Professor Leonidas Stefanis (5) and Professor Kathryn L. Evans (6), respectively. Briefly, for the luciferase assays, all transfections were performed in a 96-well plate with 0,2 µg/ well of target vector DNA 24h after $4x10^4$ cells have been seeded. Cells were Influenza A/WS/33 infected with a MOI of 1, and 24h later the luciferase activity was measured. The MOI was defined as the number of virions added per cell during infection time (7). For all experiments, at least 10 technical replicates were measured per each condition. The assay was performed according to the manufacturers recommendations (Dual-Glo®Luciferase Assay System; Promega). MDCK.2 canine cells were maintained in MEM medium supplemented with 10% (v/v) FBS, 1% (v/v) non essential amino acids (Sigma), 1% (v/v) sodium pyruvate (ThermoFisher Scientific), 1% (v/v) L-glutamine, and 1% (v/v) PenStrep upon viral infection.

Influenza A/WS/33 virus infection. LUHMES and NLF cells were infected with the influenza A/WS/33 virus strain, in serum free medium, with a MOI of 1. One hour later, the medium was replaced with normal supplemented medium, cell type required medium, and the cells were incubated for 24h until the respectively analysis was performed.

As described in the study by Tesoriero and coworkers (8), mice were instilled with 4-5 µL of the virus suspension or with phosphate-buffered saline, pH 7.4 (PBS) into one nostril for immunohistochemistry. Previously, it was found that intranasal instillation of A/WSN/33 in small volumes causes a spread to the brain in *Rag*-/- mice that is not accompanied by virus spread to the lung as determined by PCR (9). For the infection, the mice were briefly anaesthetized with isoflurane (Baxter). They were then followed daily and sacrificed, together with matched controls, before or when they showed signs of body weight loss, following the institutional guidelines and ethical protocols.

Antiviral compound administration. LUHMES cells were kept for four days in differentiation medium. Cells received 0.5 µM or 50 µM of oseltamivir phosphate (Sigma) dissolved in PBS. For the negative control conditions, cells were treated with PBS solution. The compound was applied to the cells 8h before A/WS/33 influenza infection. The dose was renewed when the new differentiation medium was added to LUHMES cells after the viral infection. All the analyses were performed 30h after the first compound dose administration.

Expression and purification of wild-type human recombinant α-synuclein.

The open reading frame of wild-type human α-synuclein was cloned into pET11a, and α-synuclein was then expressed in BL21 (DE3) Rosetta *E.coli* (Novagen). The bacteria were grown in 2YT medium (2% tryptone, 1% yeast extract and 0.5% NaCl) supplemented with

100 µg/mL carbencillin and 34 µg/mL chloramphenicol until an optical density of about 1.0 at 600 nm was reached. Expression of α-synuclein was induced with 1 mM isopropyl b-D-thiogalactoside (IPTG) for 4h at 37°C. α-synuclein was then extracted following a protocol described in ref. (10). Briefly, after centrifugation the bacterial pellet was resolved in pure water and frozen at -20°C. α-synuclein was extracted by boiling for 20 min at 95°C and then precipitated with ammonium sulfate. The α-synuclein pellets were resolved in 50 mM Tris pH 8.0 and then purified twice by ion-exchange chromatography using Q-sepharose (GE Healthcare). α-synuclein was eluted from the column with 50 mM Tris, pH8.0 and 350 mM NaCl before it was again precipitated with ammonium sulfate. The pellets were resolved in pure water and dialyzed 3x times against water (1:500) using SlideALyzer cassettes with a cut-off of 10 kDa (Thermo Scientific). Finally, α-synuclein was passed over an Amicon Ultra-15 filter with a cut off of 100 kDa (Merck) and then aliquoted and shock-frozen in liquid N2.

RT-QuiC.

Purified human wild-type α-synuclein was diluted to 70 µM in 25 mM KPO_4 pH8.0, 200 mM KCl, 10 mM NaN_3, and 100 µM ThT that was passed through a 0.22 µm filter before use. The RT-QuiC reactions were performed in black non-binding 96-well half-area microplates with flat bottom (Corning) using a Tecan Safire plate reader. Infected or non-infected LUHMES cells from one 10 cm dish were washed twice with PBS and then lysed in 500 µL hypotonic lysis buffer (10 mM Hepes pH7.6, 1.5 mM MgCl2, 10 mM KCl including protease inhibitor cocktail) by passing the cells, after 15 min of swelling on ice, five times through a 27G needle. 2 µL of cell lysates were added to 98 µL of reaction mix. After adding one glass bead (2.85-3.45 mm; Roth) in every well the plate was sealed with a plate sealer film and the samples underwent cycles of shaking (1 min, orbital shaking) and incubation for 9 min at 37°C. Fluorescence was measured every 10 min using 450 +/- 7.5 nm (excitation) and 480 +/- 7.5 nm (emission) wavelengths.

Determination of half-life time of α-synuclein and DISC1 in neuroblastoma cells

NLF neuroblastoma cells constitutively expressing DAT-V5 and inducibly expressing either full-length human DISC1 or α-synuclein were seeded at a density of $3x10^5$ per well in 6-well plates in RPMI medium (supplemented with 10% FBS, 1% PenStrep, 1% L-Glutamine). DISC1 expression was induced with 1 µg/mL doxycycline for 48h, whereas α-synuclein was induced for 5h by 500 ng/mL doxycycline. Prior to infection cells were washed with 2 mL of FBS-free RPMI medium and directly either treated with infection medium VGM (DMEM supplemented with 25 mM HEPES, 2% BSA, 1% PenStrep; all Thermo Fisher) or infected with FLUV (in VGM, MOI = 1) for 1h. Afterwards medium was aspirated and replaced with full RPMI containing 10 µM retinoic acid. At indicated time points (0h, 6h, 12h, 18h, 24h after

FLUV infection for DISC1 or 0h, 6h, 18h, 24h, 42h for α-synuclein), cells were lysed in TBST (150 mM Tris pH 7.4, 150 mM NaCl, 1% TX-100, 10 mM $MgCl_2$, 1x Protease Inhibitor, 1x PhosSTOP), subjected to DNaseI digest (1h, 37°C), protein determination by the Dc Protein Assay Kit (Bio-Rad) and then equal amounts were subjected to Western blots. The DISC1 signal was visualized by 14F2 and α-synuclein by Syn211, further antibodies were NP and actin as loading control for normalization. Each experiment was performed with 1-2 replicates per condition.

Immunocytochemistry. For immunocytochemistry analysis, cells were washed three times with PBS (Invitrogen) and fixed with 4% (v/v) PFA in PBS for 15 min, followed by three washing steps with PBS. Fixed cells were permeabilized and blocked with PBS including 0.5% (v/v) Saponin (Sigma), 5% (v/v) nonfat milk (Oxoid), 1% BSA (Sigma) for 1h at room temperature (RT). Primary antibodies (described in Table 1) were applied overnight at 4°C in blocking solution without milk. At the day after, coverslips were washed three times with PBS and incubated with Alexa-Fluor antibodies (anti-mouse IgG AlexaFluor 594; anti-rabbit IgG AlexaFluor 594; anti-mouse IgG AlexaFluor 488; anti-rabbit IgG AlexaFluor 488; Invitrogen) for 1h at RT. After washing three times with PBS and two times with water, cells were mounted with ProLong Gold with DAPI (Invitrogen), and images collected with a Zeiss Axiovision Apotome.2 confocal microscope (Zeiss).

Immunoblot. In brief, LUHMES cells were manually lysed with buffer A, containing 10 mM Tris-HCL, pH8; 150 mM NaCl; 2 mM EDTA; 0,5% Sodium Deoxycholate; 2% Nonidet P40 dissolved in water (for Western blots showed in Fig. S1B; D) or lysed with buffer B, containing 1% Nonidet P40 dissolved in PBS (for Western blots showed in Fig. 1D; 5D; 6D). Protein concentration was determined using the Dc Protein Assay Kit, and 10 µg of each sample dissolved in NuPAGE LDS Sample Buffer (including 2% β-mercaptoethanol) were applied to the gel. Novex NuPAGE SDS-PAGE Gel System (Thermo Fisher Scientific) with the corresponding NuPAGE Novex 4–12% Bis-Tris Midi Protein Gels, NuPAGE MES SDS Running buffer was used. Proteins were transferred to a nitrocellulose or PVDF membrane, and then blocked with 5% nonfat milk in PBS containing 0.05% Tween-20, for 1h at RT. Western blot analysis was performed with the correspondent antibodies described in table 1 followed by a secondary species specific antibody (IR Dye 680 or 800; LI-COR Corp.). VDAC was used as a loading control for all immunoblots performed. The membranes were scanned using the LI-COR Odyssey CLX. Band intensities were calculated from fluorescent signal using the Image Studio Version 2.1 software (LI-COR Biosciences).

Immunohistochemistry. For the immunohistochemical process, mice were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p., APL, Stockholm, Sweden), and perfused transcardially with 4% paraformaldehyde (wt/vol) with picric acid as previously described (11). Brains, trigeminal ganglia and lungs were dissected out and post-fixed in the same fixative for 90 min at 4°C, followed by rinsing in 10% sucrose (wt/vol) in 0.1 M phosphate buffer, pH 7.4, containing 0.01% sodium azide (Merck) and 0.02% bacitracin (Sigma). The tissues were kept in 10% sucrose solution for 2 days at 4°C, and then frozen with liquid carbon dioxide and sectioned on a cryostat (Microm). Serial coronal sections were collected at 12 µm thickness for trigeminal ganglia, and 20 µm for brains and lung. The sections were mounted onto superfrost plus microscope slides (Thermo Scientific) and stored at -20°C (27).

The slides were then pretreated with a Dako target retrieval solution (Agilent) prior to blocking solution, and incubated with respective primary antibodies listed in table 1. Conjugated Alexa Fluor antibodies, anti-mouse IgG AlexaFluor 594; anti-rabbit IgG AlexaFluor 594; anti-rabbit IgG AlexaFluor 488, (Invitrogen) were used as secondary antibodies and the slides were mounted with ProLong Gold with DAPI (Invitrogen). Images were taken with a Zeiss Axiovision Apotome.2 confocal microscope (Zeiss) for cells and with a Zeiss LSM880 Airyscan microscope system (Zeiss Microscopy GmbH) for tissue sections. The latter was equipped with a Plan-Apochromat 63x/1.4 oil immersion objective lens for detailed images and a Plan-Apochromat 20x/0.80 dry objective lens for overview images. For DAPI, a 405 nm laser was used as excitation and 410 – 471 nm was chosen as detection range for 20x overview images, while a BP 420-480 + BP 495-550 filter was used for Airyscan 63x micrographs. For AlexaFluor488, a 488 nm laser was used as excitation and 495 – 549 nm was chosen as detection range for 20x overview images, while a filter combination of BP 420-480 + BP 495-550 and a LP 460 was used for Airyscan 63x micrographs. For AlexaFluor594, a 561 nm laser was used for excitation and 585 – 664 nm was chosen as detection range for 20x overview images, while a BP 570-620 + LP 645 was used for Airyscan 63x micrographs. Airyscan alignment of the system was regularly checked during acquisition process and raw stacks were finally processed by the Zeiss Airyscan processing in 3D mode.

Table. Primary antibodies used for western blot (WB)/ immunocytochemistry (ICC) and immunohistochemistry (IHC) analysis

Antibody	Dilution	Source
DAT (rabbit)	1:500 (ICC)	MerckMilipore (#AB1591P)
DAT (rabbit)	1:500 (WB)	MerckMilipore (#AB2231)
TyrosineHydroxylase (mouse)	1:600 (ICC)	MerckMilipore (#AB152)
MAP2 (rabbit)	1:400 (ICC)	Synaptic Systems (#188002)
14F2 (mouse)	1:1000 (WB; ICC)	in-house, ref. (12)
DISC1 (rabbit)	1:50 (IHC)	in-house, ref. (13)
Syn211 (mouse)	1:400 (ICC)	Santa Cruz biotechnology (#sc-12767)
α-synuclein [MJFR1] (rabbit)	1:200 (ICC)/1:500(WB)	Abcam (#ab138501)
α-synuclein (sheep)	1:500(IHC)	Abcam (#ab6162)
Tau HT7 (mouse)	1:500 (WB;ICC)	Invitrogen (# MN1000)
Tau 5 (mouse)	1:200 (IHC)	Invitrogen (#AHB0042)
Phospho-Tau AT8 (mouse)	1:500 (WB)	Invitrogen (#MN1020)
Phospho-Tau AT180 (mouse)	1:400 (ICC)	Invitrogen (#MN1040)
TDP-43 (rabbit)	1:1000 (WB;ICC)	Proteintech (#10782-2-AP)
TDP43 [3H8] (mouse)	1:500 (IHC)	Abcam (#ab104223)
TDP-43 c-terminal (rabbit)	1:1000 (WB)	Proteintech (#12892-1-AP)
Influenza A NP (rabbit)	1:1000 (WB;ICC)	Invitrogen (# PA5-32242)
Influenza A/WSN/33 (rabbit)	1:100,000 (IHC)	Gift kindly provided by Dr. S. Nakajima, The Institute of Public Health, Tokyo, Japan)
LC3B (mouse)	1:1000 (WB;ICC)	Abcam (#ab48394)
LC3B (rabbit)	1:1000 (WB)	Cell Signaling (#2775)
LAMP1 [H4A3](mouse)	1:500 (WB; ICC)	DSHB by August,J.T./ Hidreth, J.E.K. (Hybridoma Produkt H4A3)
HuR [3A2] (mouse)	1:500 (ICC)	Santa Cruz biotechnology (#sc-5261)
VDAC (rabbit)	1:500 (WB)	Enzo (#ALX-210-785/1)

Statistical analysis. For quantification of protein aggregates in LUHMES and NLF neuroblastoma cell line, a systematic manually counting procedure was used. For all

immunocytochemistry data, randomized confocal images were taken in an average of 30 images from 2 independent wells per biological replicate, in a total number of, at least 3 independent biological experiments. All data were normalized against the number of cells present in the counted image. All data presented in the histograms were obtained by normalization to the correspondent non-infected condition considered 100%. Autophagosomal and lysosomal structures were quantified using 3D object counter plugin from the imageJ software Fiji. LC3 punctate dots quantified were in the range of 50-200 nm, and LAMP1 positive structures counted were in the range of 100-500 nm. GraphPad Prism (Version 5; GraphPad Software Inc., San Diego, CA, USA) was used to perform the statistical analysis. All data sets were tested for normal distribution based on the expected experimental results and appropriate non-parametric tests were chosen. Appropriate statistical tests and p-values are stated in the respective figure legends. P-values of $*P < 0.05$, $**P < 0.01$ were used as significance levels.

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Supplementary Figure legends

Fig S1. Characterization of human dopaminergic neurons (LUHMES). (A) Phase contrast microscope images of undifferentiated LUHMES (Dif 0) and at day 5 of differentiation. Scale bars 10 µm. (B) The representative immunoblot shows increased dopamine transporter (DAT) levels in LUHMES cells at differentiation day 5 compared to day 0. VDAC was used as a loading control. One of the three experiments is shown. (C) Immunostainings confirming the dopaminergic phenotype of LUHMES cells at day 5 of differentiation (green: TH, red: MAP2). (D) Immunoblot analysis of endogenous total protein levels of α-synuclein, DISC1, tau, and TDP-43 at differentiation days 0 and 5. Bar graphs showing the respective quantification of the protein levels normalized to VDAC protein, all protein levels were increased at Dif 5 compared to Dif 0. N = 3, means ± SEM. Mann-Whitney U with one-tailed Dunn's *post hoc* test was use as a statistical test (* $p < 0.05$).

Fig S2. Effects of influenza A/WS/33 (H1N1) infection on LUHMES cells. Determination of viral activity. (A) Percentage of infected LUHMES cells in a culture preparation 24h after A/WS/33 (H1N1) infection with MOI 1. N = 5, means ± SEM. (B) Viral infection titer at different time points post infection (0h, 12h, 24h) showed an increase in viral replication activity across time. Measurements were done in MDCK.2 cells infected with the culture medium supernatant of LUHMES cells infected with MOI 1 (n = 2, means ± SEM). (C-E) Co-staining of α-synuclein with thioflavin S (ThS) in three different conditions: (C) no infection, (D) A/WS/33 H1N1 infection and (E) positive control, in which preformed α-synuclein fibrils were added to LUHMES cells and stained. There is partial co-staining of α-synuclein aggregates with ThS in (D) and (E), see arrows.

Fig S3. A/WS/33 (H1N1) infection induces seeding in LUHMES cells and similar changes in α-synuclein protein homeostasis in human neuroblastoma cells. (A) Representative depiction of seeding kinetics of recombinant, purified α-synuclein in a real time quaking-induced conversion (RT-QuiC) assay using LUHMES cell lysates. As positive control, 150 nM (equivalent to α-synuclein monomers) of preformed recombinant α-synuclein fibrils were used as seed (shades of grey). Cell lysates from H1N1-infected LUHMES cells irregularly seeded (5 out of 36 reactions from 4 biological replicates) but with a delayed onset (>50 h) and different kinetics than the positive control. Non-infected LUHMES cells almost never seeded (1 out of 36 reactions from 3 biological replicates); of note, there was a low number of α-synuclein aggregates even in non-infected cells.

Infection of a different human cell line. (B) Viral infection titer (in PFU/mL) measured at different time points during 48h showed a stable viral replication activity 24h after infection.

Measurements were done in MDCK.2 cells infected with the culture medium from infected NLF neuroblastoma cells (means ± SEM). (C) Aggregated α-synuclein was detected in the cytoplasm of NLF neuroblastoma cells transiently transfected with human α-synuclein after 24h of influenza infection (MOI 1). Aggregated α-synuclein was not detected in non-infected neuroblastoma cells (green: α-synuclein, red: influenza A NP, blue: DAPI). Scale bars 10 μm.

Fig S4. A/WS/33 (H1N1) infection induces DISC1 aggregation in human cells. (A) Non-infected LUHMES cells did not show DISC1 aggregates (green: DISC1, blue: DAPI). (B) DISC1 aggregates were detected in the cytoplasm of LUHMES cells 24h after influenza infection (MOI 1; green: DISC1, red: influenza A NP, blue: DAPI). Scale bars 10 μm. (C) Viral infection (black bar) induced DISC1 aggregates as presented by percent DISC1 aggregates per cell compared to non-infected cells (grey bar). N = 4, means ± SEM. Mann-Whitney U with one-tailed Dunn's *post hoc* test was used as statistical test (*p <0.05). (D) 24h of influenza infection (MOI 1) induced DISC1 aggregation in NLF neuroblastoma cells transiently transfected with human DISC1. In non-infected cells no aggregates were visible (green: mRFP-DISC1, red: influenza A NP, blue: DAPI). Scale bars 10 μm. (E) LUHMES cells did not show α-synuclein and DISC1 co-aggregation 24h after influenza infection (MOI 1); green: α-synuclein, red: DISC1, blue: DAPI). Scale bars 10 or 3 μm.

Fig S5. A/WS/33 (H1N1) infection increases α-synuclein and DISC1 half-life time in inducible neuroblastoma cell lines. (A) Western blot of NLF cell lysates infected (+) or non-infected (-) with influenza A H1N1 WS/33 (MOI = 1, +) following a 5h induction of α-synuclein expression led to increased signal intensity of α-synuclein compared to controls over a time course of 42h post infection. (B) Normalized α-synuclein levels as determined by densitometry of infected cells as percent of non-infected controls are shown, dotted line indicates respective non-infected control at 100%. To maximize the infection effect on α-synuclein levels for analysis, the mean values of α-synuclein signals at the tested time points were added and a t-test against the sum of means of the non-infected condition was performed (t-test against a hypothetical value as statistical test, n = 4, originating from 4 x 100% of time points 6h, 18h, 24h, 48h = 400). This showed a significant increase in α-synuclein signal intensity following A/WS/33 infection (p = 0.027). (C) Western blot of NLF cell lysates infected (+) or non-infected (-) with influenza A H1N1 WS/33 48h after induction of DISC1 expression (MOI = 1). Compared to non-infected controls the DISC1 signal was stronger in the infected condition at the indicated time points after infection, showing that DISC1 half-life time was increased in response to A/WS/33 infection. A representative blot with 2 technical replicates per condition is shown. (D) Densitometric measurement of normalized DISC1 protein levels with (+) and without (-) influenza A H1N1 WS/33 infection.

By calculating the percent signal change in A/WS/33-infected vs. non-infected cells, an effect of virus on the prolongation of DISC1 half-life time was shown. One-way ANOVA ($p = 0.037$) with one-tailed Dunnet's *post hoc* test (0h, 6h, 12h, 18h: n.s., 24h: $p < 0.011$). The dotted line indicates respective non-infected control at 100%. In analogy to S5B, the mean values of DISC1 signal after infection as % of non-infected control was added up ($n = 5$). A t-test against a hypothetical value of 500 ($n = 5$, therefore 5 x 100% for time points 0h, 6h, 12h, 18h, 24h = 500), showed a strong significant increase in DISC1 signal intensity following A/WS/33 infection ($p < 0.006$). Abbreviations: h, hours; n.s., not significant. (E) Influenza A/WS/33 (H1N1) infection does not affect SNCA promoter activity. 24h of influenza infection (MOI 1) did not induce changes in the activity of the SNCA 5'-promoter. A luciferase reporter assay was performed in NLF neuroblastoma cells transiently transfected with pLG3- SNCA 5'-promoter and infected with influenza for 24h (MOI 1). N = 3, means ± SEM. Mann-Whitney U with one-tailed Dunn's *post hoc* test was used as statistical test. (F) Influenza A/WS/33 (H1N1) infection does not affect DISC1 promoter activity. NLF neuroblastoma cells were transiently transfected with pLG4.10- DISC1 promoter and 24h after influenza-infection a luciferase reporter assay was performed. N = 3, means ± SEM. Mann-Whitney U with one-tailed Dunn's *post hoc* test was used as statistical test.

Fig S6. Influenza A/WS/33 (H1N1) infection does not induce cytoplasmic TDP-43 aggregates or tau protein changes in human dopaminergic neurons. (A) Cytoplasmic inclusions of TDP-43 were not detected in LUHMES cells 24h after influenza infection (MOI 1). Viral infection did not induce TDP-43 positive stress granules (green: TDP-43, red: influenza A or stress granule marker HuR). Scale bars 10 µm. (B) 24h of infection did not induce changes in phosphorylation pattern of tau or in total tau in LUHMES cells (green: phospho-tau AT180 or total tau HT7, red: influenza A NP, blue: DAPI). Scale bars 10 µm. (C) LUHMES cells stained with fluorescent secondary antibodies only (green: Alexa 488, red: Alexa 594, blue: DAPI). Scale bars 10 µm

Fig S7. Characterization of potentially aggregation-prone proteins in LUHMES cells. Co-staining of α-synuclein with thioflavin S (ThS) in different conditions. Upper panels, no infection; lower panels, A/WS/33 infection. Blue: DAPI, red: ThS signal, green: (A) DISC1 (B) total tau (HT7) (C) phospho-tau (AT8) (D) TDP-43. There was no co-staining observed.

Fig S8. 28 days of influenza A/WSN/33 (H1N1) infection changes mouse Disc1 expression *in vivo.* (A) Increased mouse Disc1 protein levels were detected in A/WSN/33 (H1N1) infected brain region of *Rag1* knock-out mice in comparison with the non-infected animals (PBS control) (green: mDisc1, red: influenza A HA, blue: DAPI). Scale bars 10 µm.

(B) Secondary antibody control stains showed no cross-reactivity (green: Alexa 594 secondary antibody, red: Alexa 488 secondary antibody or influenza A HA, blue: DAPI).

Fig S9. A/WSN/33 (H1N1) infection does not affect protein homeostasis of Tdp-43 or tau *in vivo*. Similar levels of mouse Tdp-43 and total tau in non-infected compared to A/WSN/33 (H1N1)-infected *Rag1* knock-out mice. (A) Non-infected mice (green: Tdp-43, red: influenza A HA, blue: DAPI). (B) Non-infected mice (green: HT7 recognizing total tau, red: influenza A HA, blue: DAPI). (C) Infected area of an A/WSN/33 (H1N1)-infected *Rag1* knock-out mouse (green: Tdp-43, red: influenza A HA, blue: DAPI). (D). Infected area of an A/WSN/33 (H1N1)-infected *Rag1* knock-out mouse (green: HT7 recognizing total tau, red: influenza A HA, blue: DAPI). Scale bars 50 μm, 5 μm.

Fig S10. Influenza A virus (A/WS/33) segment 5 nucleocapsid protein induces lower DISC1 aggregation levels. (A) NLF neuroblastoma cells were double transfected with human DISC1 and influenza virus segment 5 nucleocapsid. Only basal levels of DISC1 aggregates comparable to non-infected control were detected upon influenza virus segment 5 nucleocapsid transfection (green: mRFP-DISC1, red: influenza A NP; blue: DAPI). Scale bars 10 μm. (B) Quantification of percentage of aggregated DISC1 per cell infected with A/WS/33 virus (MOI 1, black bar) and transfected with A/WS/33 nucleocapsid (light grey bar) relative to the non-infected condition (dark grey bar). N = 3, means ± SEM. One-way ANOVA test was used as a statistical test (n.s., non-statistical significance).

24h of influenza A virus (A/WS/33) infection leads to a decrease in the fusion of autophagosomes with the acidified proteolytic lysosomes. (C) Immunostainings of autophagosome marker LC3 and lysosomal marker LAMP1 showed a decrease in co-localization of LC3-LAMP1 in influenza-infected LUHMES cells (24h, MOI 1) in comparison with non-infected condition (green: LC3, red: LAMP1, blue: DAPI). Scale bars 10 μm. (D) Quantification of percentage of endogenous LC3 co-localizing with LAMP1 in infected condition (black bar) relative to the non-infected condition (grey bar). Viral infection led to a decrease in LC3/ LAMP1 colocalization. N = 3, means ± SEM. Mann-Whitney U with one-tailed Dunn's post hoc test was used as statistical test * $p < 0.05$.

Figure S1

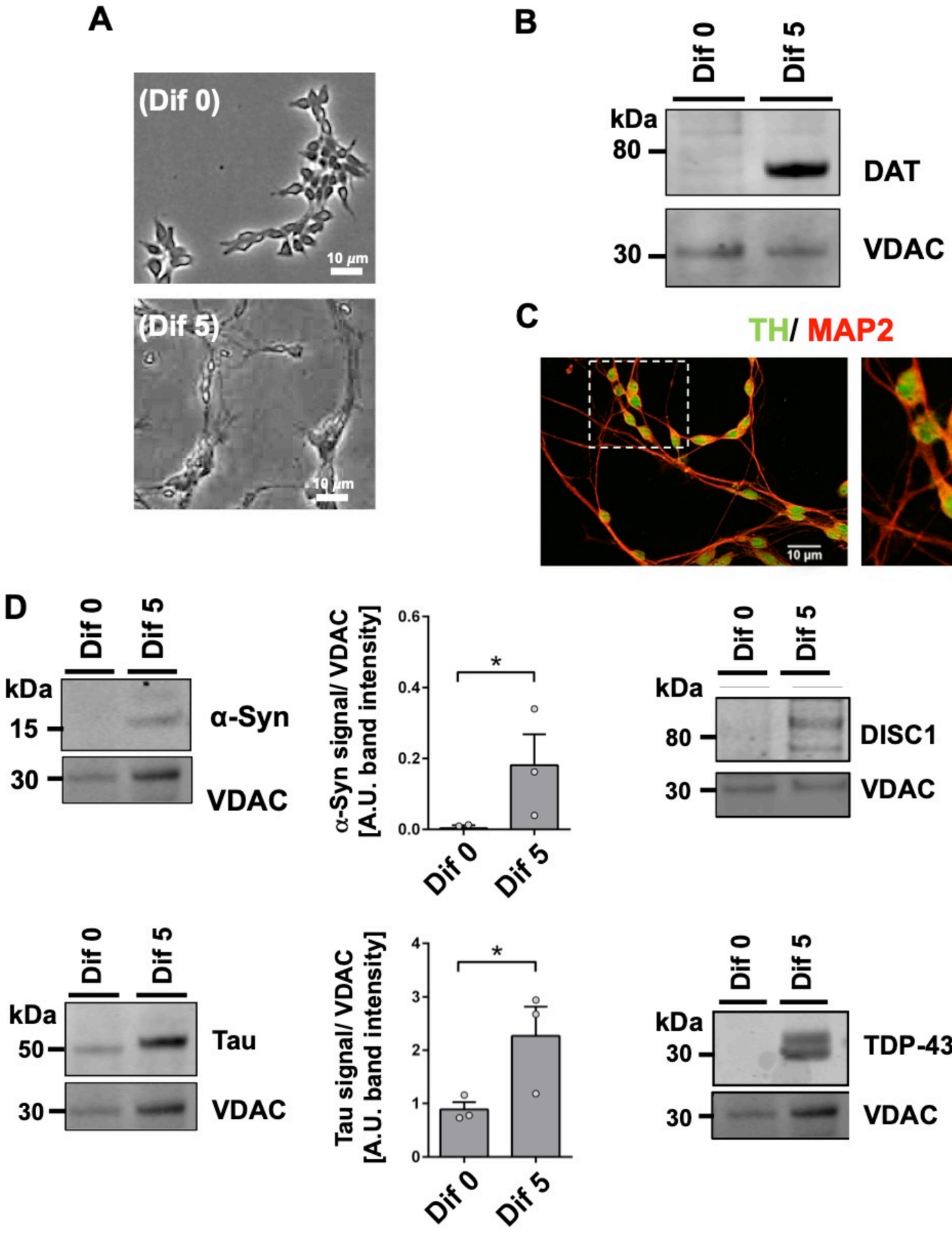
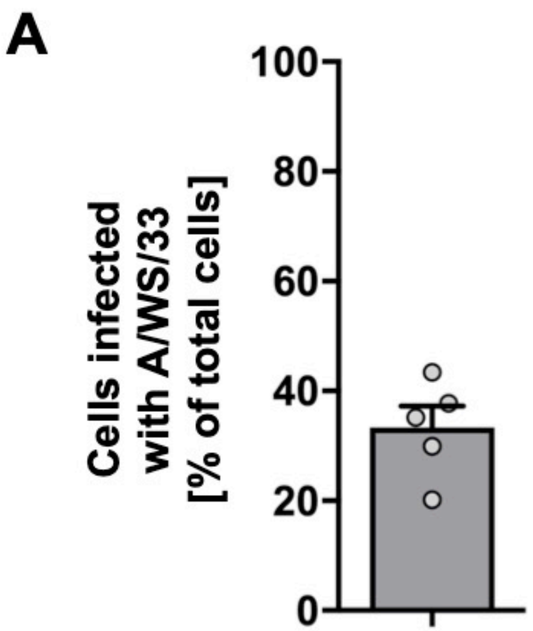
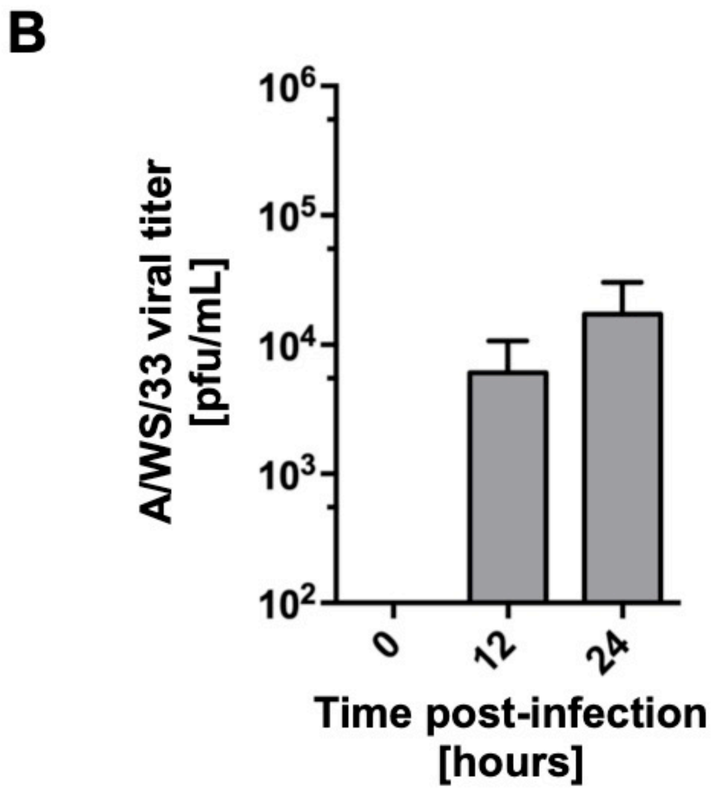

A
(Dif 0)
10 μm
(Dif 5)
10 μm
B
Dif 0
Dif 5
kDa
80
DAT
30
VDAC
C
TH/ MAP2
10 μm
D
Dif 0
Dif 5
kDa
15
α-Syn
30
VDAC
α-Syn signal/ VDAC [A.U. band intensity]
0.6
0.4
0.2
0.0
*
Dif 0
Dif 5
kDa
80
DISC1
30
VDAC
DISC1 signal/ VDAC [A.U. band intensity]
0.10
0.08
0.06
0.04
0.02
0.00
*
Dif 0
Dif 5
kDa
50
Tau
30
VDAC
Tau signal/ VDAC [A.U. band intensity]
4
3
2
1
0
*
Dif 0
Dif 5
kDa
30
TDP-43
30
VDAC
TDP-43 signal/ VDAC [A.U. band intensity]
10
8
6
4
2
0
*
Dif 0
Dif 5

Figure S2


A
Cells infected with A/WS/33 [% of total cells]
100
80
60
40
20
0


B
A/WS/33 viral titer [pfu/mL]
10^6
10^5
10^4
10^3
10^2
0
12
24
Time post-infection [hours]

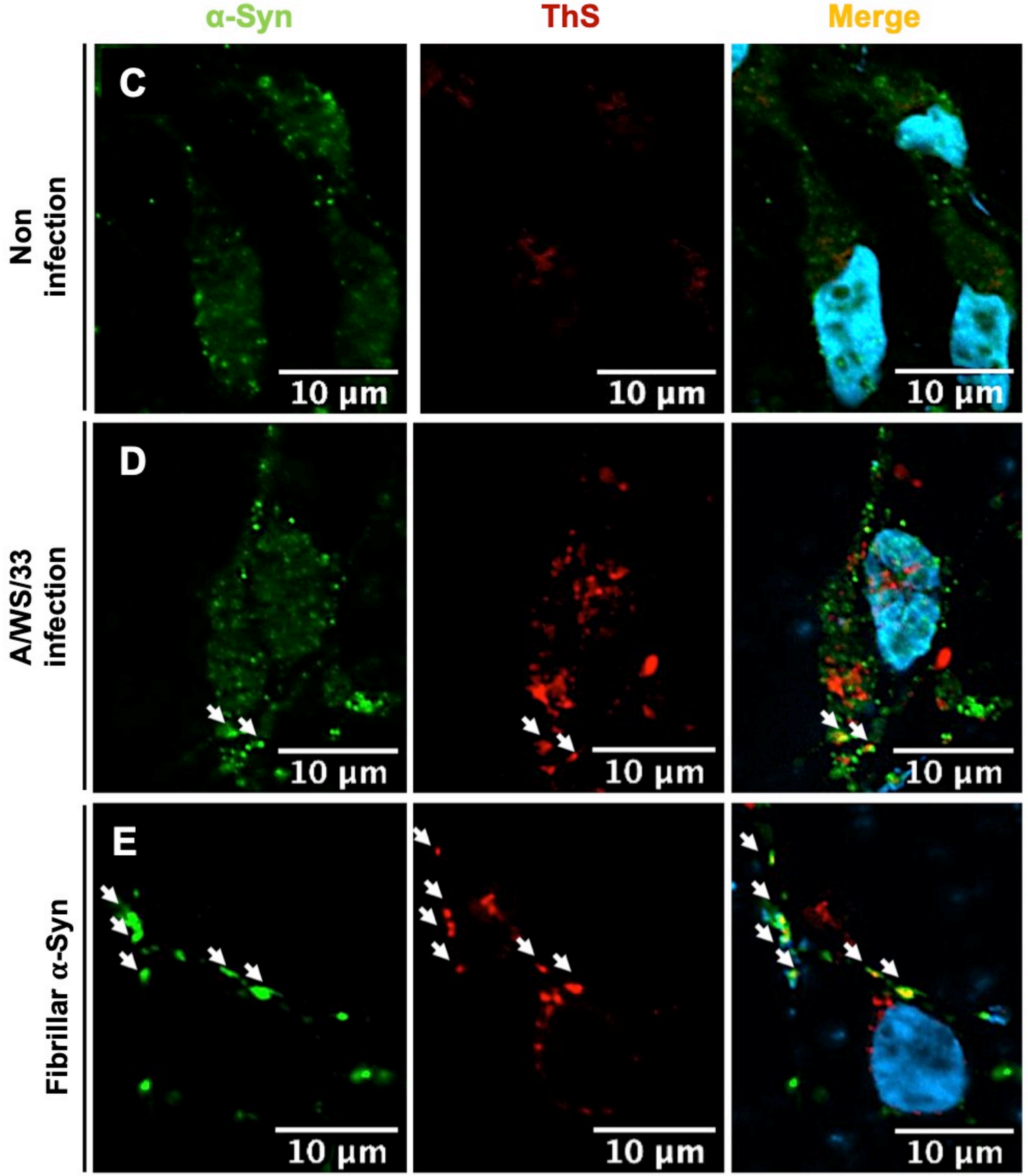

α-Syn
ThS
Merge
Non infection
A/WS/33 infection
Fibrillar α-Syn
C
D
E
10 µm

Figure S3

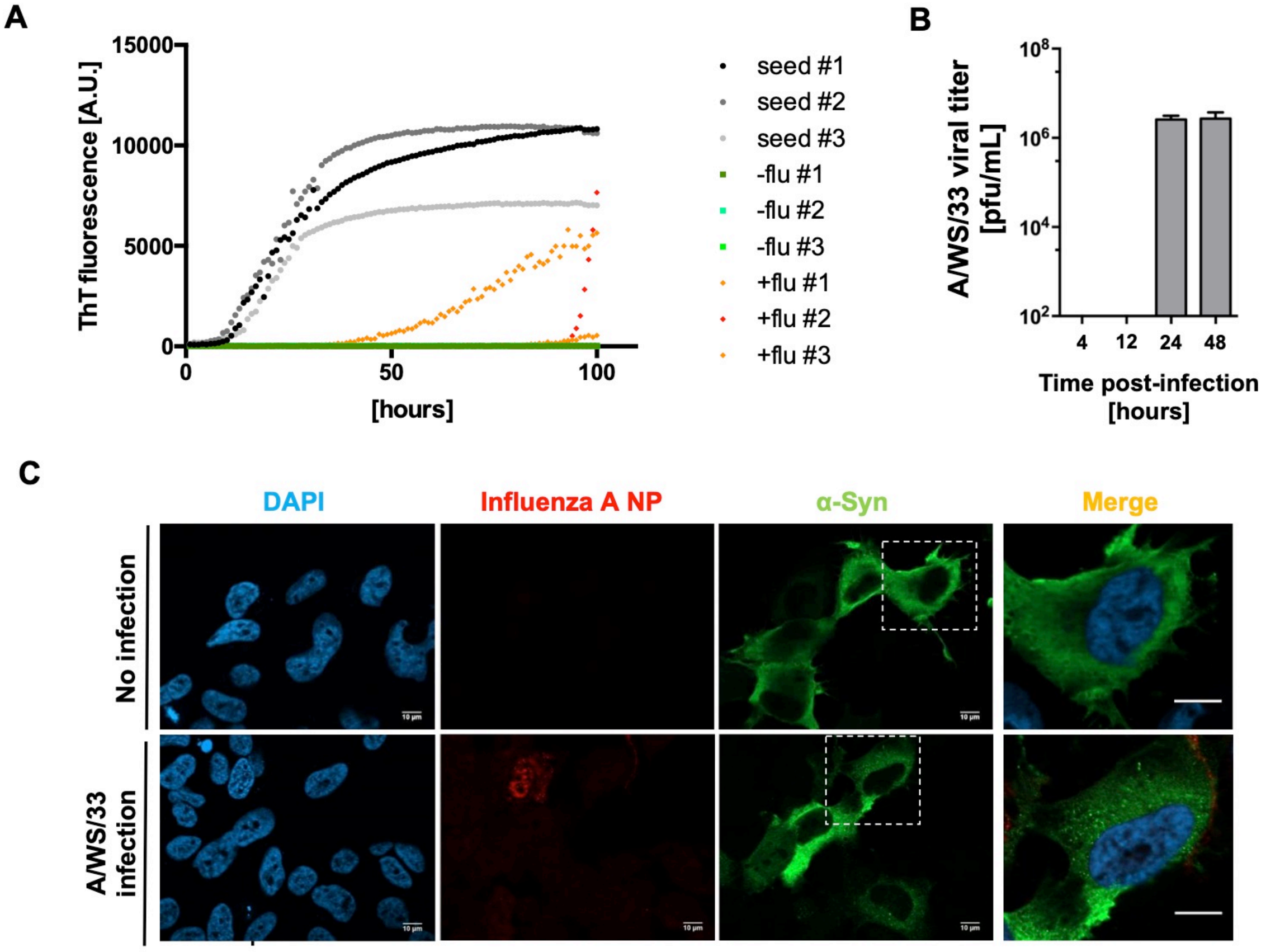
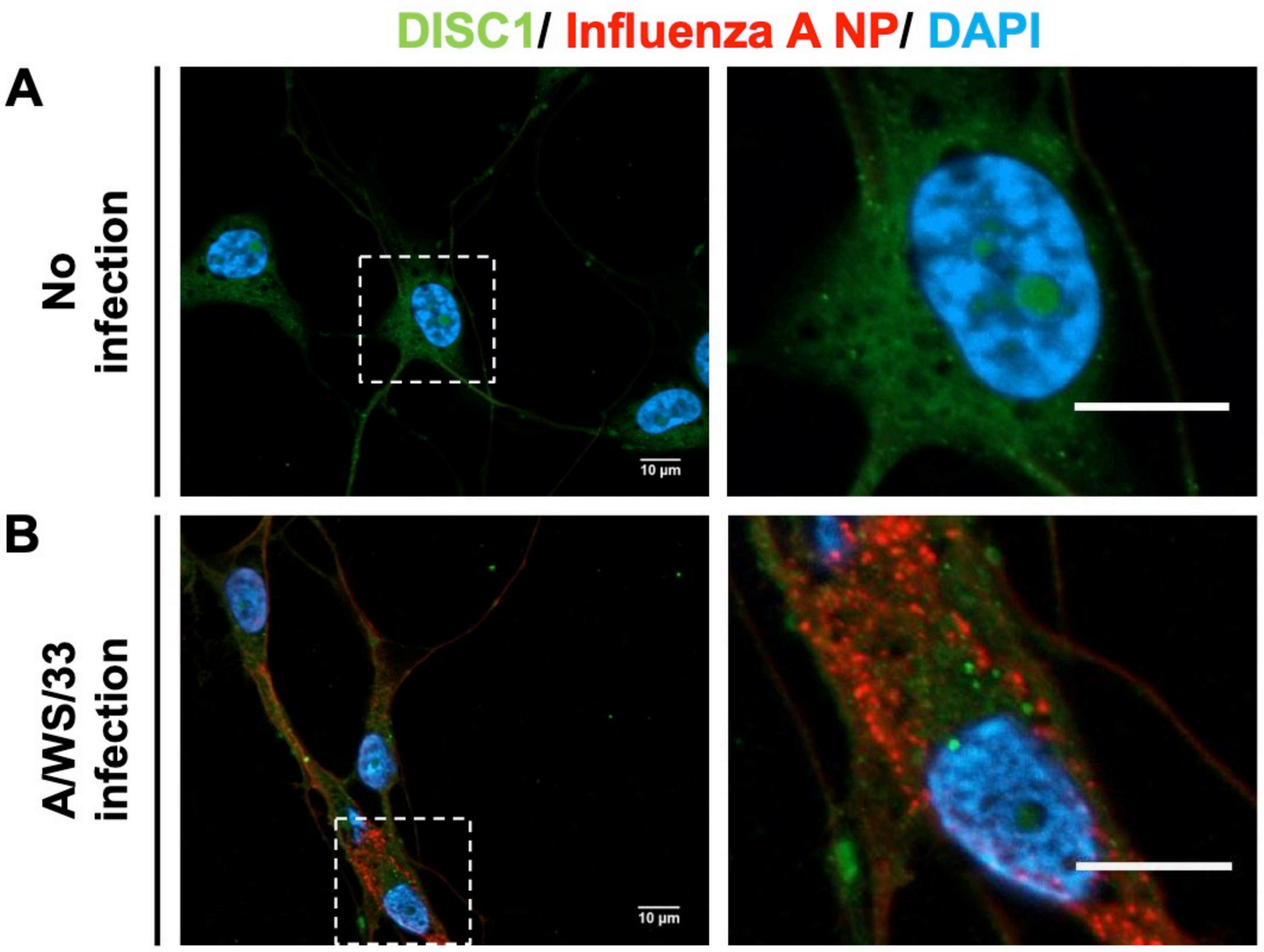
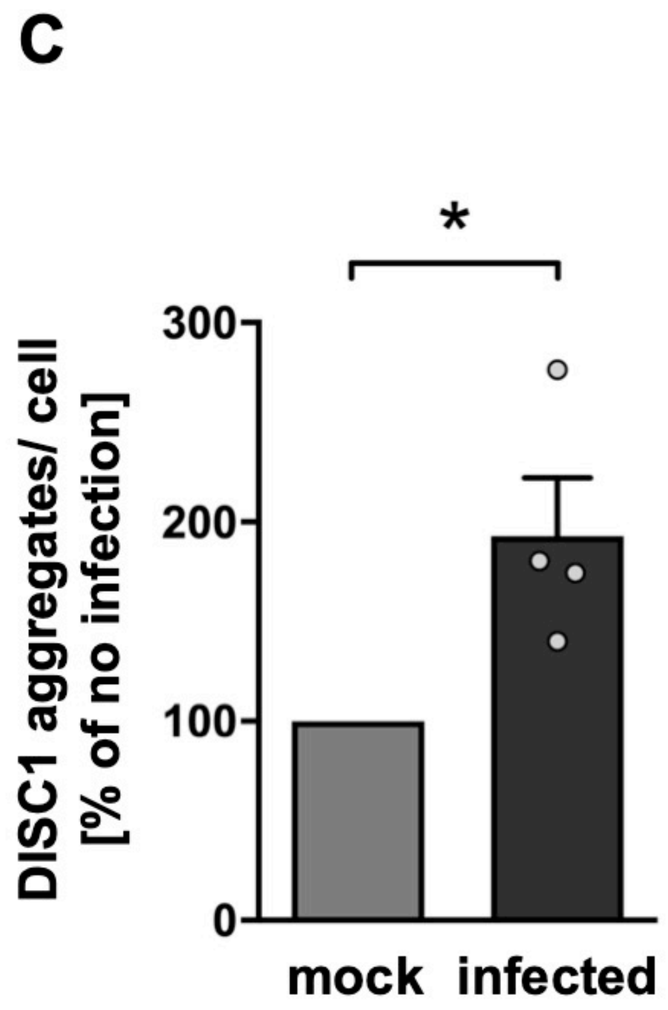
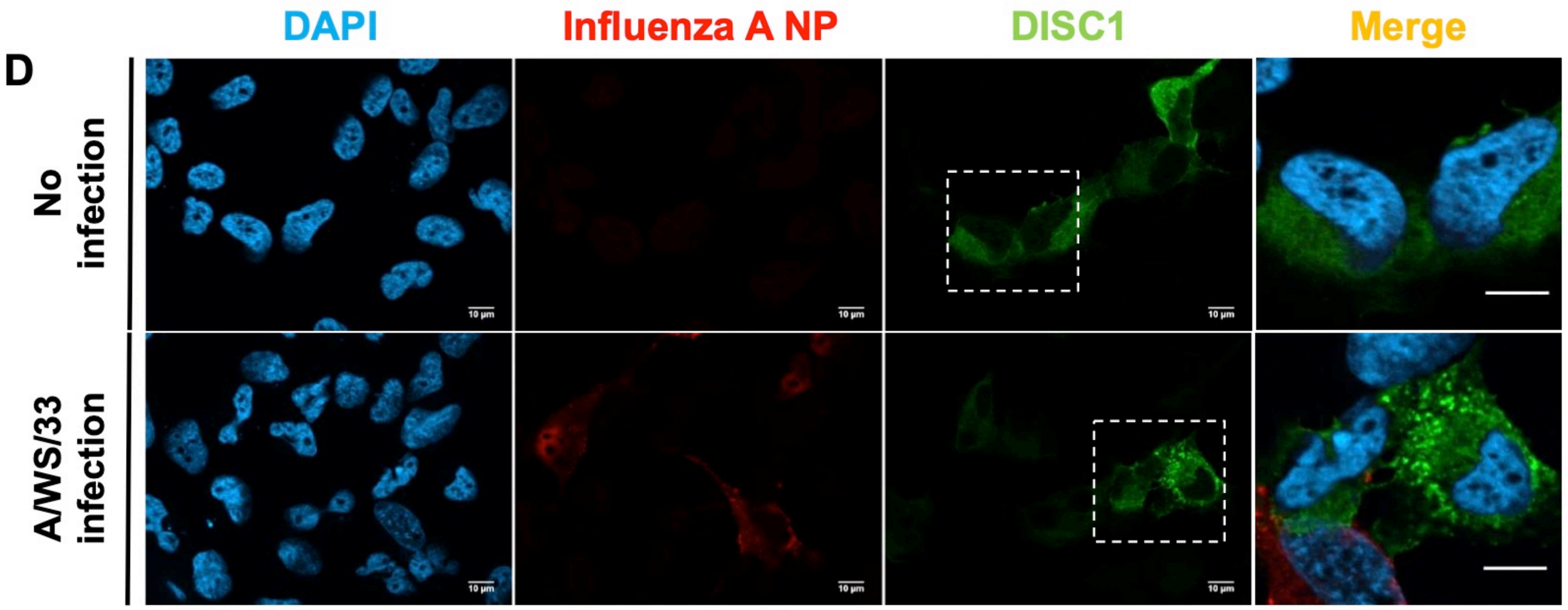

A
ThT fluorescence [A.U.]
15000
10000
5000
0
0
50
100
[hours]
seed #1
seed #2
seed #3
-flu #1
-flu #2
-flu #3
+flu #1
+flu #2
+flu #3
B
A/WS/33 viral titer [pfu/mL]
10^8
10^6
10^4
10^2
4
12
24
48
Time post-infection [hours]
C
DAPI
Influenza A NP
α-Syn
Merge
No infection
A/WS/33 infection
10 μm

Figure S4


DISC1/ Influenza A NP/ DAPI
A
No infection
B
A/WS/33 infection
10 µm


C
*
300
200
100
0
DISC1 aggregates/ cell [% of no infection]
mock
infected


DAPI
Influenza A NP
DISC1
Merge
D
No infection
A/WS/33 infection
10 µm

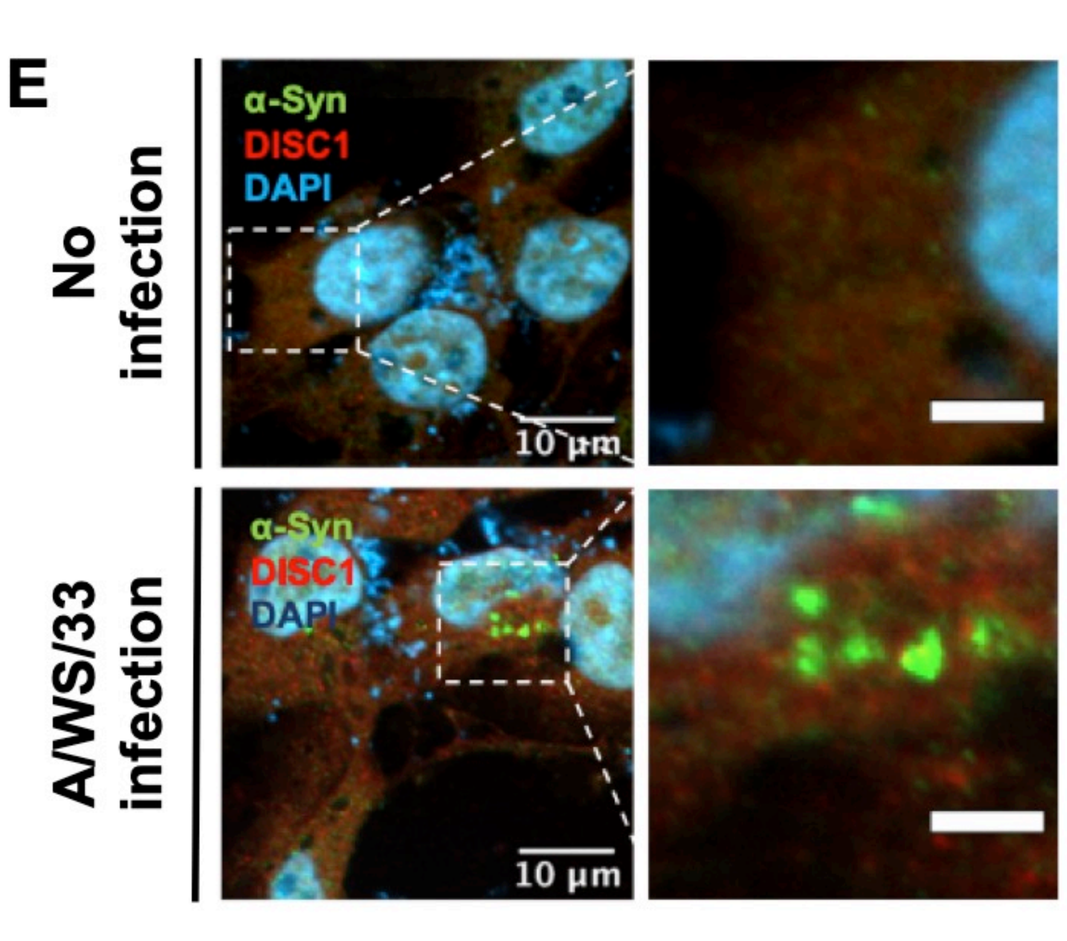
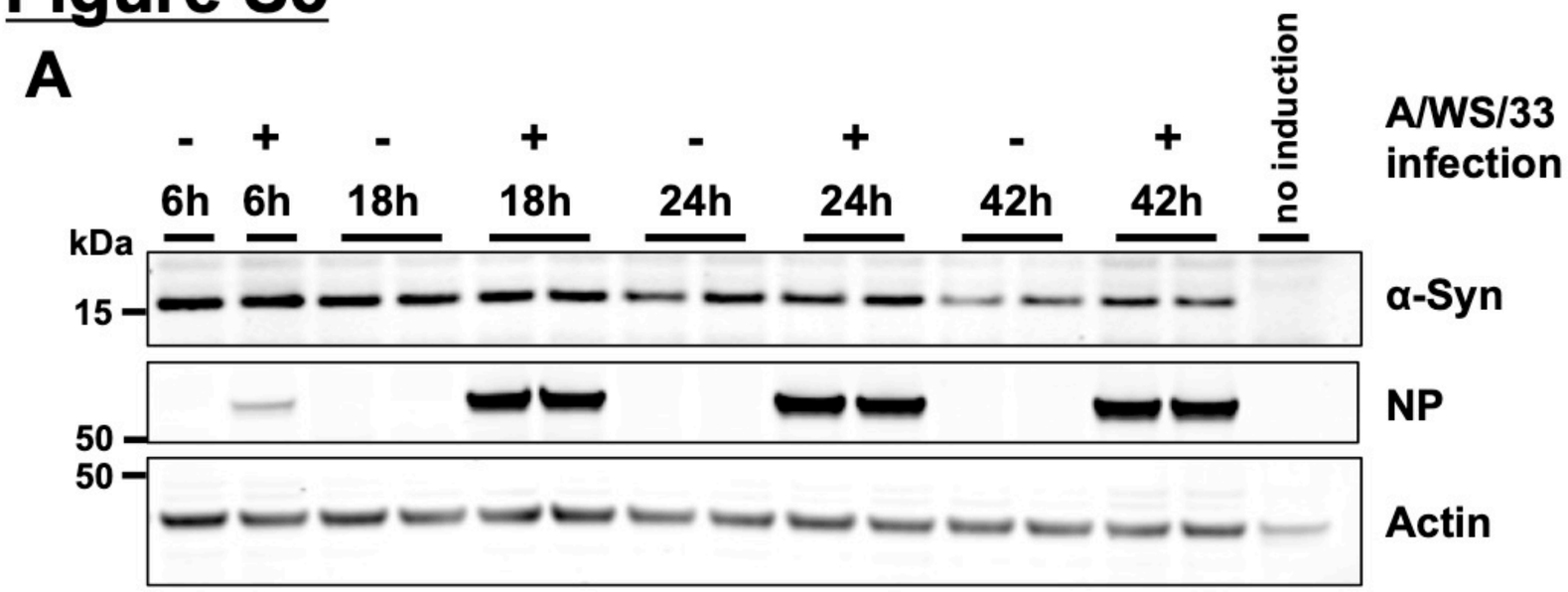
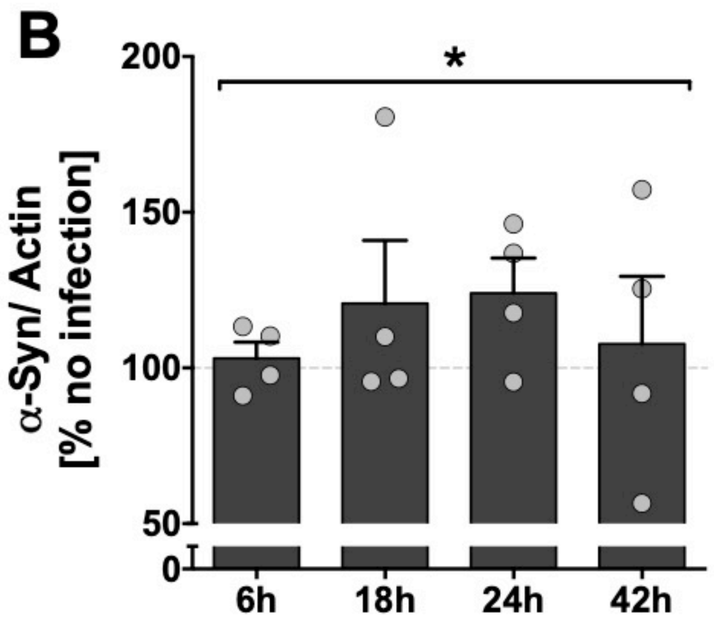
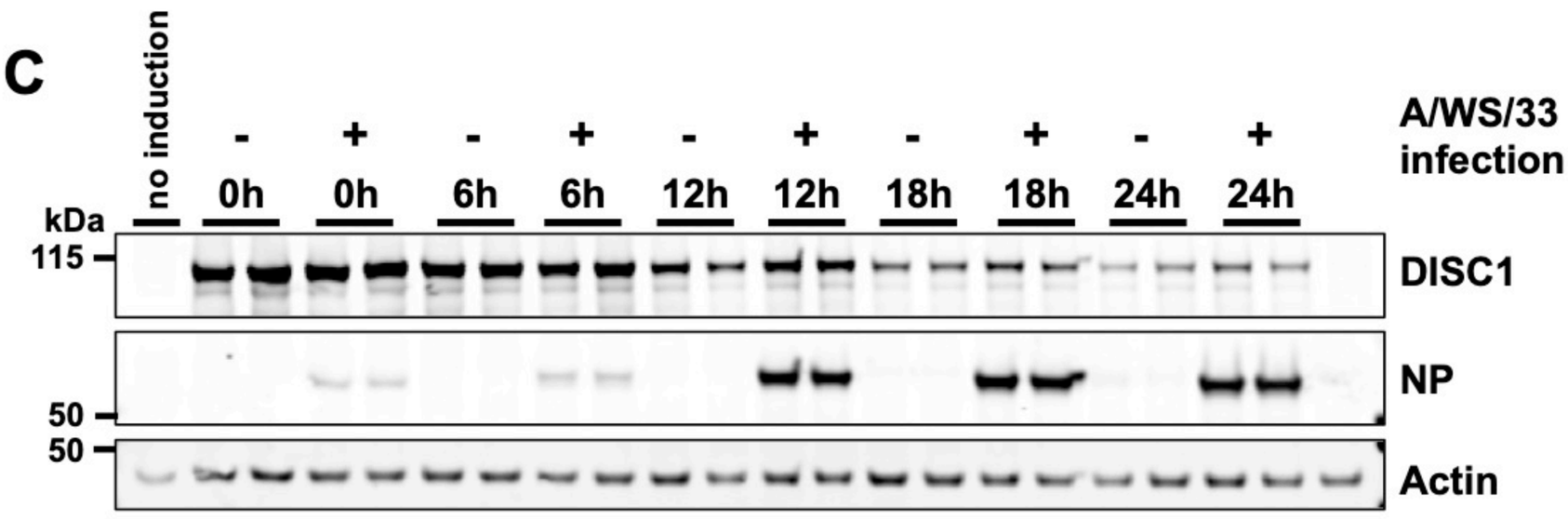
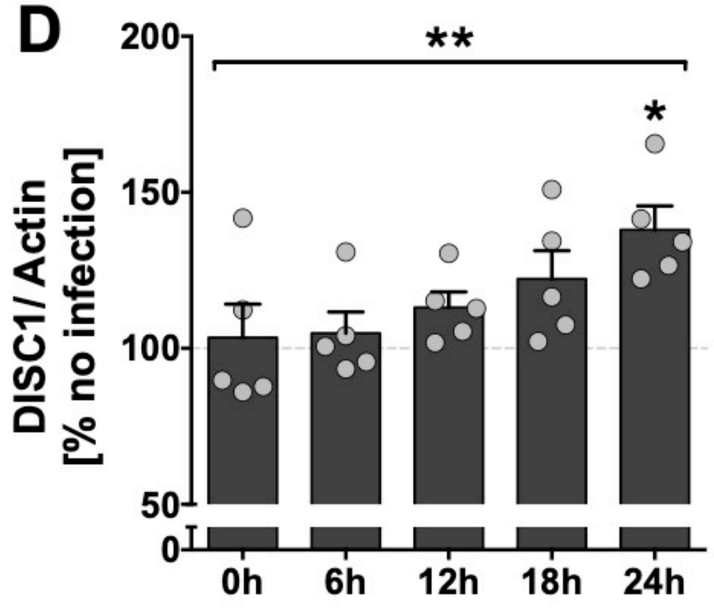

E
No infection
A/WS/33 infection
α-Syn
DISC1
DAPI
10 µm

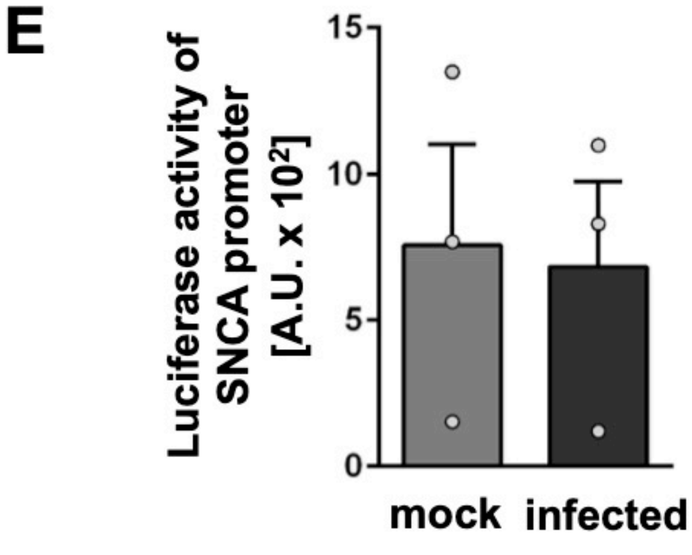
Figure S5


A
A/WS/33 infection
- + - + - + - +
6h 6h 18h 18h 24h 24h 42h 42h
no induction
kDa
15
50
50
α-Syn
NP
Actin


B
α-Syn/ Actin [% no infection]
200
150
100
50
0
*
6h
18h
24h
42h


C
A/WS/33 infection
no induction
- + - + - + - + - +
0h 0h 6h 6h 12h 12h 18h 18h 24h 24h
kDa
115
50
50
DISC1
NP
Actin


D
DISC1/ Actin [% no infection]
200
150
100
50
0
**
*
0h
6h
12h
18h
24h


E
Luciferase activity of SNCA promoter [A.U. x 10²]
15
10
5
0
mock
infected

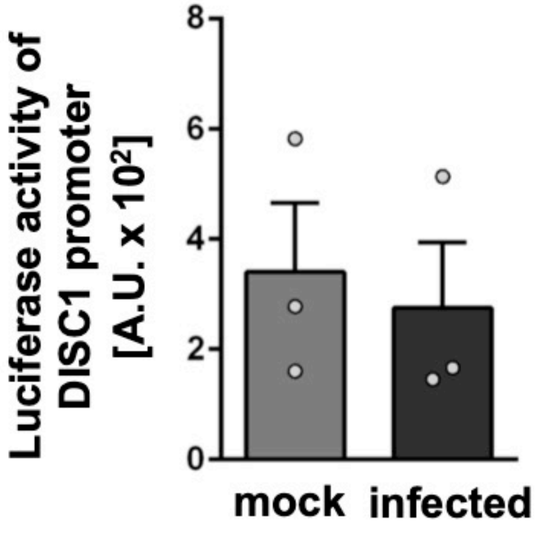
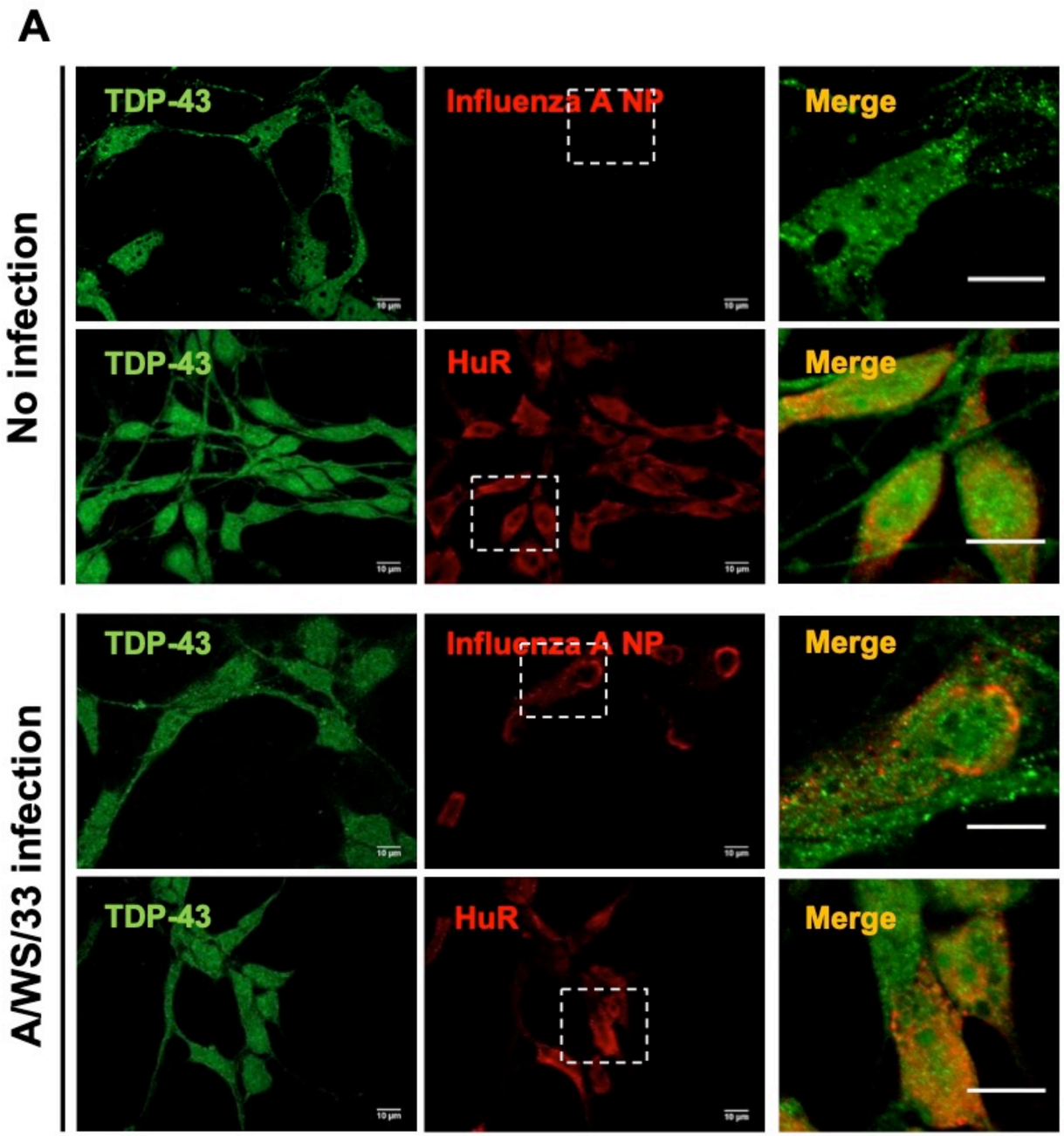
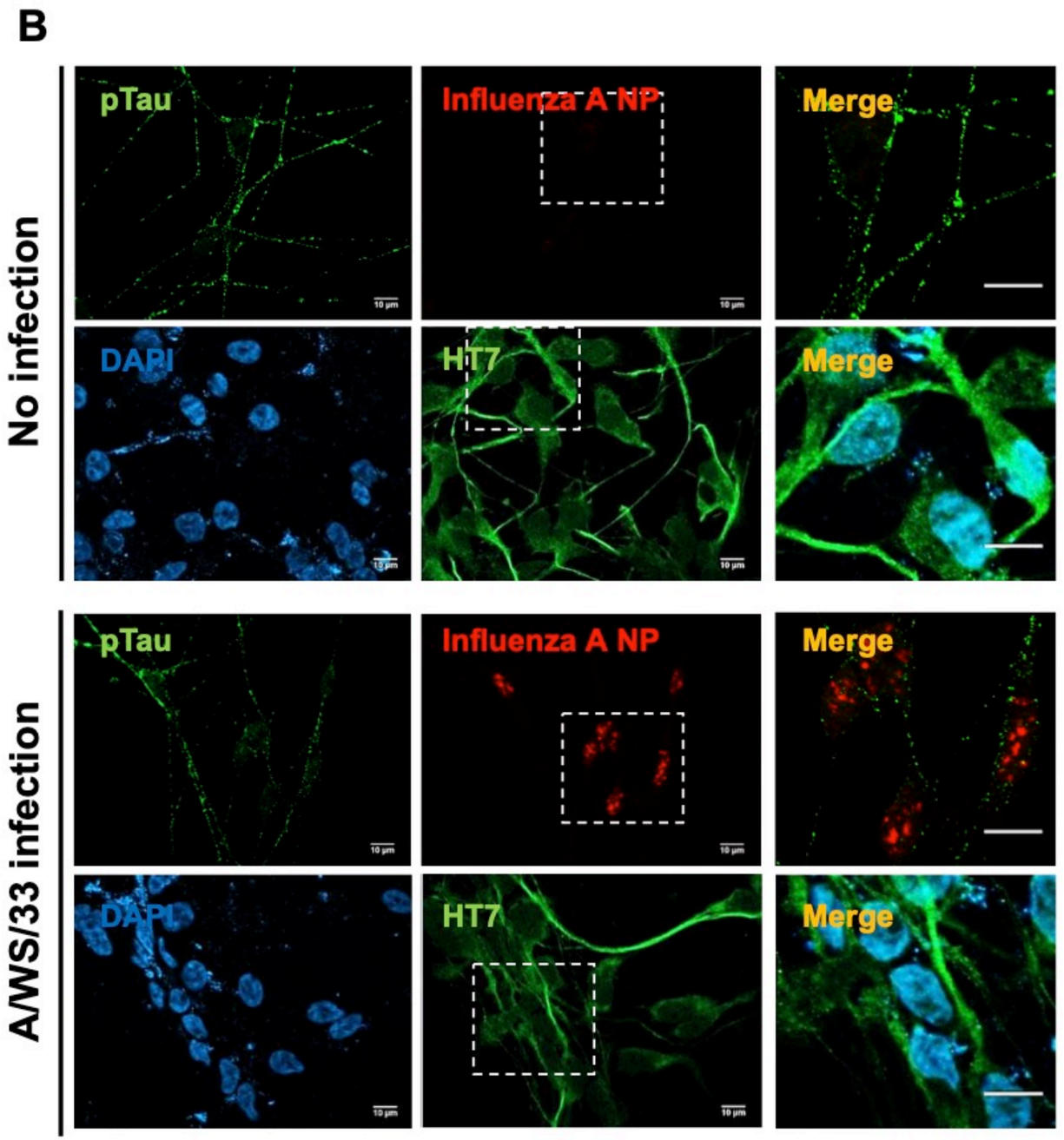

F
Luciferase activity of DISC1 promoter [A.U. x 10²]
8
6
4
2
0
mock
infected

Figure S6


A
No infection
TDP-43
Influenza A NP
Merge
TDP-43
HuR
Merge
A/WS/33 infection
TDP-43
Influenza A NP
Merge
TDP-43
HuR
Merge


B
No infection
pTau
Influenza A NP
Merge
DAPI
HT7
Merge
A/WS/33 infection
pTau
Influenza A NP
Merge
DAPI
HT7
Merge

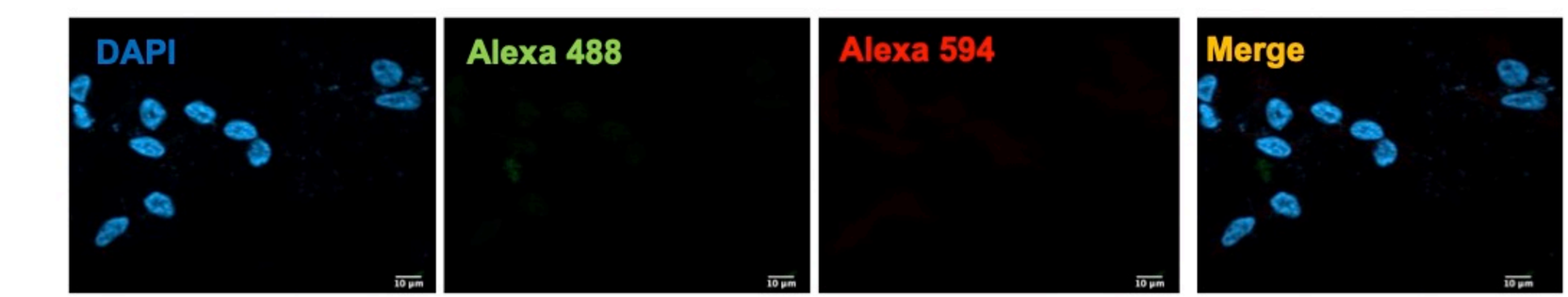

C
DAPI
Alexa 488
Alexa 594
Merge

Figure S7

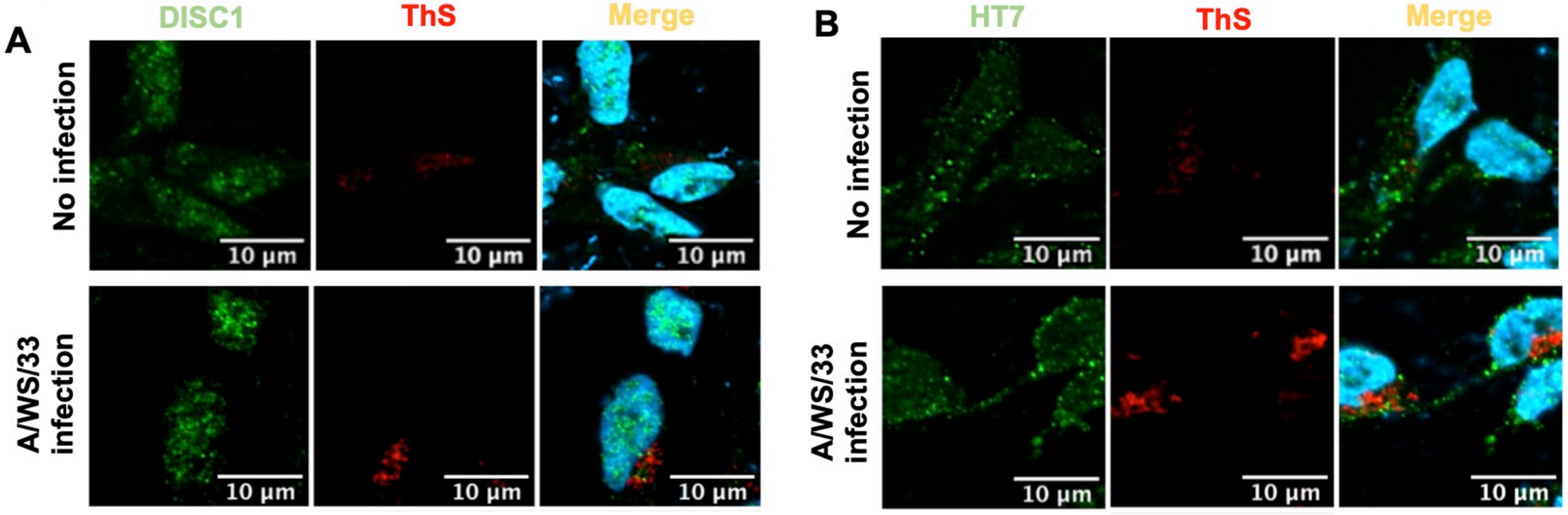

A
DISC1
ThS
Merge
No infection
A/WS/33 infection
10 μm
B
HT7
ThS
Merge
No infection
A/WS/33 infection
10 μm
C
TDP-43
ThS
Merge
No infection
A/WS/33 infection
10 μm
D
p-tau
ThS
Merge
No infection
A/WS/33 infection
10 μm

Figure S8

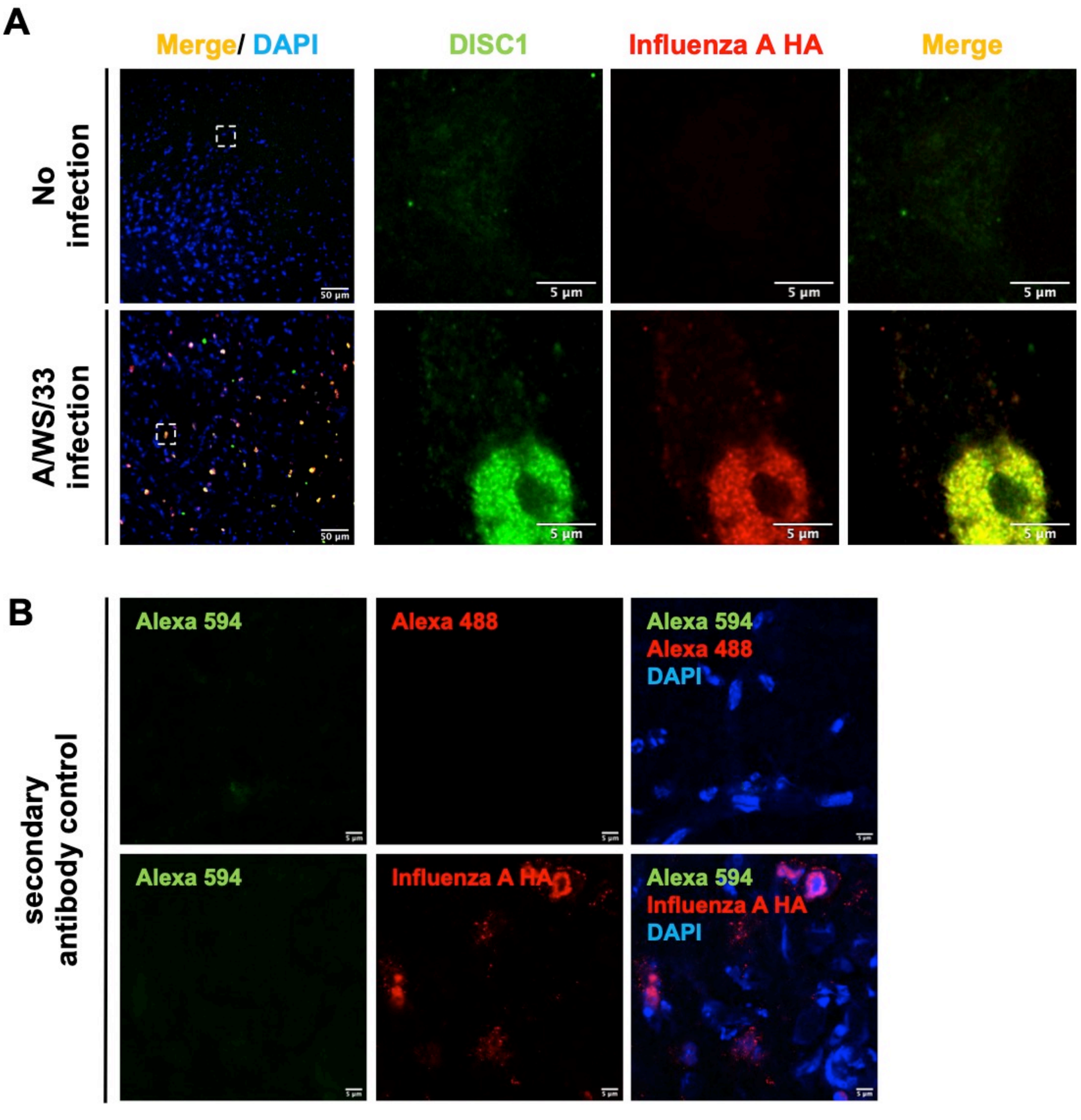

A
Merge/ DAPI
DISC1
Influenza A HA
Merge
No infection
A/WS/33 infection
50 µm
5 µm
B
secondary antibody control
Alexa 594
Alexa 488
Alexa 594
Alexa 488
DAPI
Alexa 594
Influenza A HA
Alexa 594
Influenza A HA
DAPI

Fig S9A in editable format

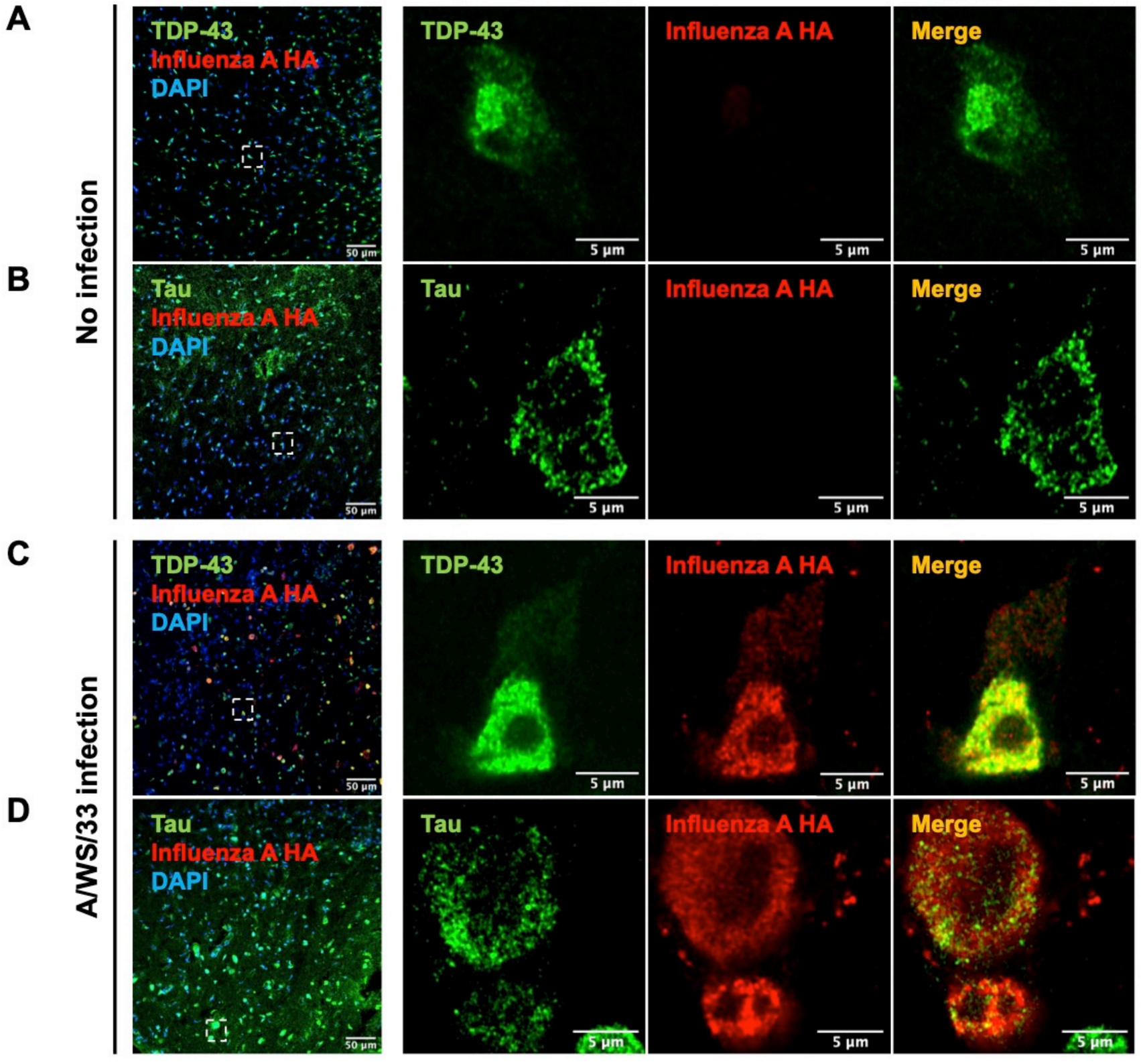
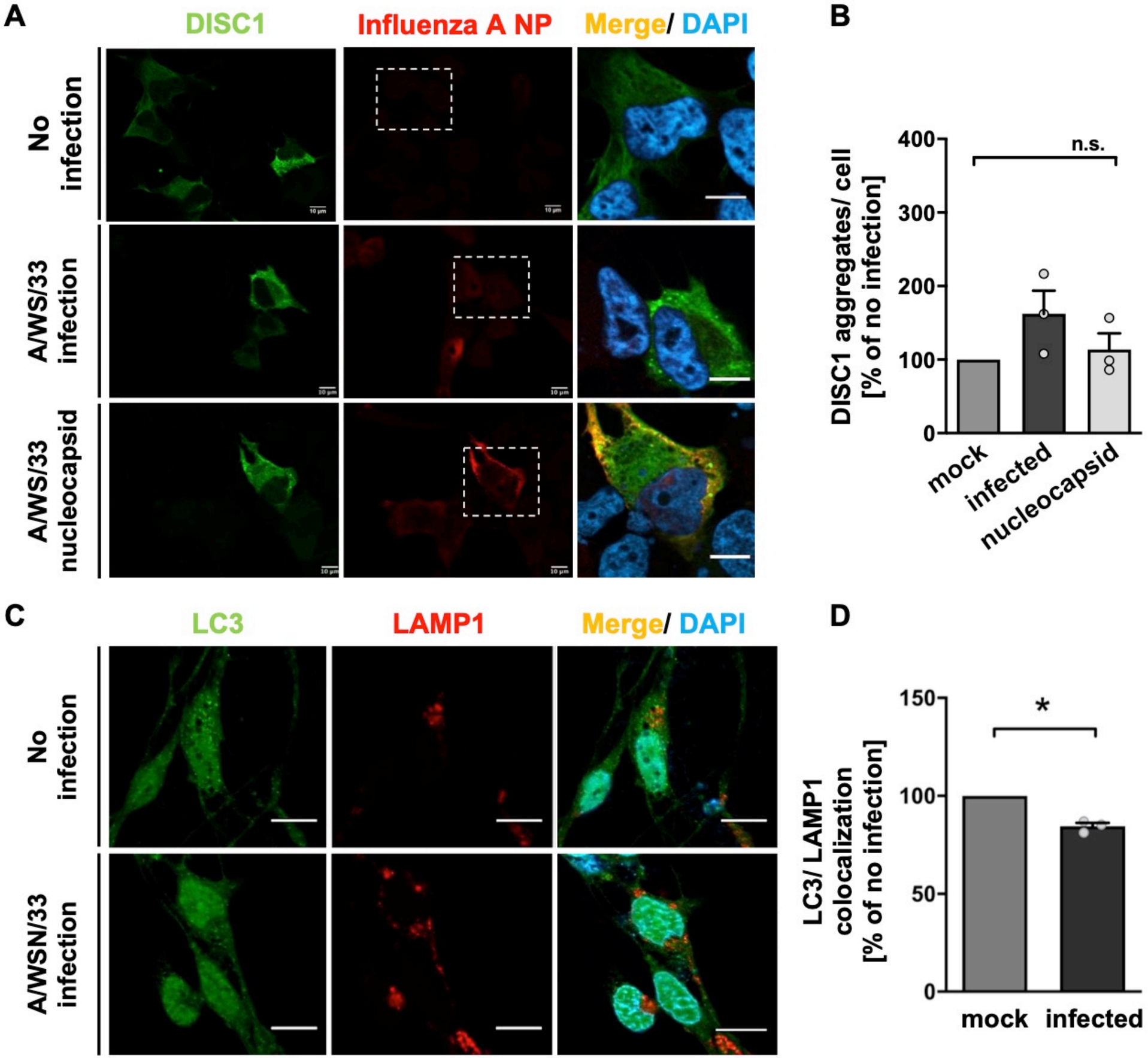


Figure S10


A
DISC1
Influenza A NP
Merge/ DAPI
No infection
A/WS/33 infection
A/WS/33 nucleocapsid
B
DISC1 aggregates/ cell [% of no infection]
400
300
200
100
0
n.s.
mock
infected
nucleocapsid
C
LC3
LAMP1
Merge/ DAPI
No infection
A/WSN/33 infection
D
LC3/ LAMP1 colocalization [% of no infection]
150
100
50
0
*
mock
infected