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RESEARCH ARTICLE

Dissection of mammalian orthoreovirus μ 2 reveals a self-associative domain required for binding to microtubules but not to factory matrix protein μ NS

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Abstract

Mammalian orthoreovirus protein μ 2 is a component of the viral core particle. Its activities include RNA binding and hydrolysis of the γ -phosphate from NTPs and RNA 5'-termini, suggesting roles as a cofactor for the viral RNA-dependent RNA polymerase, λ 3, first enzyme in 5'-capping of viral plus-strand RNAs, and/or prohibitory of RNA-5'-triphosphate-activated antiviral signaling. Within infected cells, μ 2 also contributes to viral factories, cytoplasmic structures in which genome replication and particle assembly occur. By associating with both microtubules (MTs) and viral factory matrix protein μ NS, μ 2 can anchor the factories to MTs, the full effects of which remain unknown. In this study, a protease-hypersensitive region allowed μ 2 to be dissected into two large fragments corresponding to residues 1–282 and 283–736. Fusions with enhanced green fluorescent protein revealed that these amino- and carboxyl-terminal regions of μ 2 associate in cells with either MTs or μ NS, respectively. More exhaustive deletion analysis defined μ 2 residues 1–325 as the minimal contiguous region that associates with MTs in the absence of the self-associating tag. A region involved in μ 2 self-association was mapped to residues 283–325, and self-association involving this region was essential for MT-association as well. Likewise, we mapped that μ NS-binding site in μ 2 relates to residues 290–453 which is independent of μ 2 self-association. These findings suggest that μ 2 monomers or oligomers can bind to MTs and μ NS, but that self-association involving μ 2 residues 283–325 is specifically relevant for MT-association during viral factories formation.

Introduction

The M1 genome segment of mammalian orthoreovirus (MRV) encodes the 83-kDa μ 2 protein, one of five protein components of the MRV core particle [1–4]. The core, with ~20 copies of μ 2 [5], is released into the cytoplasm as the “payload” of cell entry [6, 7]. Once in the cytoplasm, it mediates transcription, 5'-capping and export of full-length plus-strand RNAs

templates by the ten double-stranded RNA (dsRNA) genome segments [4, 8]. These transcripts are then used for translation of the MRV proteins or as templates for full-length minus-strand RNA synthesis and packaging to generate new dsRNA segments within newly assembling core particles. At least some of the newly assembled cores also produce plus-strand transcripts, either before or instead of undergoing outer-capsid assembly to become infectious virions, substantially by amplifying the levels of MRV transcripts and proteins produced in infected cells [9–12].

Although the precise roles of μ 2 in RNA synthesis remain uncertain, the purified protein acts as a nucleoside triphosphatase (NTPase) as well as an RNA 5'-triphosphatase (RTPase) [13]. In the same lines, there is evidence showing that the μ 2-encoding M1 genome segment is a genetic determinant of MRV strain differences in *in vitro* NTPase activities of cores, resulting in critical dependence of viral replication [14, 15]. The M1 genome segment is also the genetic determinant of strain differences in *in vitro* transcription yields and responses to different temperatures by cores [16] as well as in sensitivity of MRV growth to mycophenolic acid, which decreases cellular pools of GTP [17]. A temperature-sensitive mutant that renders μ 2 defective at supporting MRV genome synthesis in cells suggests that newly synthesized μ 2 may also be a component of the replicase complex or otherwise involved at an early step in viral replication [18]. Results from knockdown of μ 2 expression by RNA interference are consistent with this conclusion [19, 20]. Together, these findings suggest roles for μ 2 in RNA synthesis in both mature (transcribing) and nascent (assembling and genome-replicating) cores. Interaction of μ 2 with the viral RNA-dependent RNA polymerase (RdRp) λ 3 has been demonstrated with the two purified proteins [13]. Purified μ 2 also binds RNA [21], which may be further necessary for its roles in cores.

Perhaps relating to its roles in nascent cores, μ 2 is a component of viral factories [22, 23], which are the sites of MRV genome replication and particle assembly in cells. The MRV nonstructural protein μ NS forms the matrix of these factories, and μ 2 has been shown to associate with μ NS in infected or co-transfected cells [23, 24]. A minimal region of μ NS both necessary and sufficient for the μ 2 association has been defined [14, 24, 25], but the regions of μ 2 involved in μ NS association remain unknown.

MRV factories can be observed by light microscopy and have two basic morphologies, globular or filamentous [23, 26–29]. When expressed in the absence of other MRV proteins, μ NS forms factory-like structures (FLS) that are morphologically similar to globular factories. Remarkably, the μ 2 protein of certain MRV strains, including Type 1 Lang (T1L) in co-expression with μ NS redirect the FLS to microtubules (MTs), generating filamentous structures [24]. In fact, the single expression of μ 2 protein associate and stabilize MTs in cells [23], and purified T1L μ 2 showed to co-sediment with purified MTs *in vitro* [13]. The globular morphology of factories formed by a few other MRV strains [23, 28, 29] has been attributed to point mutations in μ 2, as in the case of our lab's version of strain Type 3 Dearing (T3D^N), which contains a the substitution of a Ser by Pro at residue 208 (S208P). When expressed in the absence of μ NS, T3D^N μ 2 shows a reduced capacity to associate with MTs and tends to form small aggregates [23, 29, 30]. Complementation of μ 2 *in trans* after silencing of core-transcribed M1 mRNAs by RNA interference has provided direct evidence that MT association by μ 2 is essential for MRV growth in at least some settings [19]. However, the regions of μ 2 involved in MT association remain unknown. Interestingly, the residue 208 mediates repression of IFN- β signaling, modulating MRV induction of IFN- β in mice cardiac myocytes [31]. IFN- β regulation by μ 2 has been associated with the presence of a conserved sequence present among MRV strains, denoted as immunoreceptor tyrosine-based activation motif (ITAM). This motif is present in the μ 2 amino acid (aa) region 118–134, and it is related to the activation of NF- κ B and the recruitment of Syk kinase intermediate into the viral factories [32]. Also, T1L μ 2 can

retain IRF9 in the nucleus, a protein associated with the innate immune response suppression of T1L strains but not of T3D strains [33, 34]. The presence of a nuclear localization signal motif in μ 2 residues 99 to 110 [14] could also play a role in this pathway, as it may be a requirement for μ 2 nuclear import and further suppression of the host innate immune response.

More complex phenotypes associated with MRV infections are also genetically influenced by M1/ μ 2. These phenotypes include (i) the capacity of MRV T1L to grow more efficiently than T3D in primary myocardial cells [35]; (ii) the ability of T1L, but not T3D, to grow effectively in endothelial and Madin-Darby canine kidney cells by favoring progeny assembly, with viral fitness analysis suggesting that cell tropism variations can be due to a critical role related to aa 347 [36–38]; (iii) the greater capacity of MRV variant 8B to cause myocarditis in mice, correlating phenotypes in cultured cardiac myocytes [39–41]; (iv) the largest capacity of T1L than T3D to grow and cause lesions in the liver, coupled with the stronger virulence of T1L with respect to T3D, in severe-combined-immunodeficient mice [42]. These M1-influenced differences in growth efficiency and virulence between MRV strains suggest that μ 2 may interface with host factors to influence the outcome of infection in particular cells or tissues. This aspect could be accomplished in several ways if: (i) the RTPase activity of μ 2 prohibits RNA-5'-triphosphate-activated antiviral signaling pathways [43–45] or (ii) the MT-association activity of μ 2 affects MRV growth, spread, or effects on cells [19, 23].

Given that there are many unsolved questions regarding the roles of μ 2 in MRV growth and effect of host cells, we were interested in functionally dissecting the determinants present in the μ 2 protein. In this study, we used fluorescence microscopy as a tool for gauging the associations of μ 2 with MTs and itself, as well as the relationship between these two activities. Following the same methodology, we establish the minimal requirement of μ 2 to associate with the matrix forming protein, μ NS into FLS. The results obtained expand our understanding of the functional organization of μ 2 by identifying N-terminal regions of the protein that are essential for all three activities.

Materials and methods

Cells, viruses, and antibodies

CV-1 (African green monkey kidney fibroblast, M.L. Nibert own collection, HMS) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone) and 10 μ g/ml gentamicin (Invitrogen). MRV strains T1L and T3D^N were derived from the Bernard N. Fields lab. Rabbit polyclonal antisera specific for μ 2 and μ NS were used as described previously [23, 46]. Mouse antibody (MAb) anti-acetylated α -tubulin (clone 6-11B-1) and mouse MAb anti- α -tubulin (clone B-5-1-2) were obtained from Sigma-Aldrich. Rabbit polyclonal anti-tubulin (H-300) and mouse MAb anti-GFP (B-2) were obtained from Santa Cruz Biotechnology, Inc. Rabbit polyclonal anti-GFP (ab290) was obtained from Abcam. Mouse MAb HA.11 was obtained from Covance; goat anti-rabbit immunoglobulin G (IgG) conjugated to Alexa 594, goat anti-mouse IgG conjugated to Alexa 594, and goat anti-mouse IgG conjugated to Alexa 488, were obtained from Molecular Probes, Invitrogen.

Proteolytic digestion and N-terminal sequencing of partially purified μ 2

Baculovirus-based expression and partial purification of μ 2(T1L) were performed as described previously [13]. Proteolytic treatment was carried out at room temperature in a 10- μ l volume with 5 μ g of μ 2 and 0.5 μ g of Thermolysin (Sigma-Aldrich) in digestion buffer (20 mM Tris acetate (pH 8.5), 50 mM KCl, 5 mM MgCl₂, 10% glycerol, 2 mM β -mercaptoethanol). The reaction was stopped by addition of 0.5 μ l of 0.5 M EDTA pH 8.0. For N-terminal sequencing,

samples were loaded onto 10% deionized polyacrylamide mini gels. The gel was pre-run for 10 min at 80 mA with 2 mM thioglycolic acid (Fisher Scientific) in the upper buffer (125 mM Tris (pH 6.8), 0.1% SDS). Samples were denatured by mixing with gel sample buffer (final concentrations: 125 mM Tris (pH 8.0), 10% sucrose, 1% SDS, 2% 2-mercaptoethanol, 0.01% bromophenol blue) and incubated for 30 min at 65°C. After this treatment, the samples were loaded and separated at 100 V for stacking gel and at 300–350 V through the resolving gel. Proteins were transferred to Immobilon-PSQ polyvinylidene fluoride membrane (Millipore) at 4°C for 50 min at 80 V. The membrane was washed in distilled water for 10 min, stained with Coomassie brilliant blue R-250 (Sigma-Aldrich) for 5 min, and destained twice in 50% methanol for 5 min and twice in water for 5 min. The membrane was air dried and mailed to Midwest Analytical, Inc., for N-terminal sequencing via Edman degradation.

Oligonucleotides and plasmid constructions

The oligonucleotides were obtained from Invitrogen and are listed in [S1](#) and [S2](#) Tables. A detailed description of the plasmid constructions is provided in the supporting information ([S1 File](#)).

Fluorescence microscopy

CV-1 cells (5×10^5 /well) growing in six-well plates (9.6 cm^2 /well) containing round glass coverslips (diameter, 18 mm) were transfected with 4 μg of plasmid and 10 μl of Lipofectamine 2000 (Invitrogen), according to manufacturer's instructions. At 20 h post transfection (hpt), the cells were fixed for 10 min in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1 mM KH_2PO_4 (pH 7.5)) containing 2% paraformaldehyde (PFA) or when indicated, for 3 min in cold methanol at -20°C , permeabilized for 5 min in PBS containing 0.1% Triton X-100, and blocked in PBS containing 1% bovine serum albumin for 30 min. All steps were performed at room temperature. For immunofluorescence, primary and secondary antibodies were diluted in PBS containing 1% bovine serum albumin and incubated for 45 min at room temperature in a humid chamber. Nuclei were stained with 70 nM 4,6-diamino-2-phenylindole (DAPI) (Molecular Probes). Cells were mounted in Prolong Gold (Molecular Probes) and images were acquired using a Nikon Eclipse TE 2000-U fluorescence microscope or confocal laser-scanning microscope (Leica, DM 5500 Q) equipped with a 63 X 1.3 oil objective. Data were analyzed with Leica Application Suite (Mannheim, Germany) and the Imaris software package (Bitplane, Switzerland). Collected images were processed and optimized with Photoshop (Adobe Systems) or with ImageJ Version 1.42q (National Institutes of Health, USA). For counts, at least 100 cells were individually analyzed for each phenotype. Images were prepared for publication using PowerPoint (Microsoft) software.

Cytoplasmic platform assay for protein-protein associations

The assay was performed as described previously [[47–50](#)]. Briefly, CV-1 cells growing at 5×10^5 cells/well in six-well plates were transfected as described above with 10 μl of Lipofectamine 2000, 4 μg of “fish” plasmid and 1 μg of “bait” plasmid. At 20 hpt, cells were fixed in PBS containing 2% PFA for 10 min at room temperature. Nuclei were stained with 70 nM DAPI. Cells were mounted in Prolong Gold and observed in a Nikon Eclipse TE 2000-U fluorescence microscope. Collected images were processed and optimized with Photoshop or with ImageJ version 1.42q. Cells were scored as positive or negative concerning the localization of “fish” protein to the FLS platforms formed by “bait” protein.

Pull down assay

For each experimental point, 3×10^6 CV-1 cells were infected with recombinant vaccinia virus strain VVT7.3 [51] [MOI, 3 pfu/cell] for 1 h at 37°C and then transfected with 9 μ g of DNA plasmid using Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer instructions. At 15 hpt, media was replaced by complete media containing 10 μ M MG132 (Sigma-Aldrich) and incubated for 2 h at 37°C. Before lysis, cells were washed once with PBS, incubated with 600 μ M DSP (dithiobis(succinimidyl propionate), Thermo Fisher Scientific) in PBS for 15 min on ice and washed three times with 50 mM Tris/HCl pH 8.0, 150 mM NaCl. Cells were lysed in TNN buffer (100 mM Tris pH 8.0, 250 mM NaCl, 0.5% NP-40 and cOmplete protease inhibitor cocktail (Roche, Switzerland)) for 10 min on ice and centrifuged at 15000 x g for 7 min at 4°C. The supernatant (cellular extract) was recovered and mixed with 50 μ l of Perfect-Pro Ni-NTA agarose (5 PRIME, Germany) equilibrated in 25 mM imidazole in PBS for 2 h at 4°C in a wheel. The flow through was recovered by centrifuging at 3000 x g for 2 min at 4°C. The resin was washed with 10 volumes of 35mM imidazole in PBS and eluted by incubation for 30 min in a wheel at 4°C with 100 μ l of 250 mM imidazole in PBS. Samples (cellular extract, flow through and elution) were analyzed by immunoblotting as previously described [52].

Sequence alignments

Alignments of μ 2-homolog proteins were performed using the program T-Coffee [53] at <http://www.ebi.ac.uk/t-coffee/>. The following sequences were analyzed (GenBank accession number listed for each): MRV T1L μ 2 (AF461682), MRV Type 2 Jones μ 2, (AAK54567), MRV T3D μ 2 (AF461683), MRV Type 1 Clone 29 μ 2 (AAR96289), MRV T1 Netherlands 1984 μ 2 (AAR96290), MRV Type 2 Simian Virus 59 μ 2 (AAR96292), MRV Type 3 Clone 44 μ 2 (AAR96294), ARV 138 μ A (AY557188), ARV 176 μ A (AY557189), ARV S1133 μ A (AY639610), ARV 918 μ A (AY639617), ARV Muscovy duck reovirus S14 μ A (ABJ80882), AqRV grass carp reovirus (AF450324), AqRV Golden shiner reovirus (AF403402), and AqRV golden ide reovirus (AF450324). All available sequences with unique variations in the region aligning with μ 2 residues 290–307 are shown in the discussion.

Results

Protease-hypersensitive region near residue 280 in MRV μ 2 protein

After partial purification and storage at 4°C [13], the 83-kDa μ 2 protein of MRV T1L was slowly degraded into two fragments, \sim 50,000- M_r (50K) and \sim 30,000- M_r (30K), as assessed by SDS-PAGE (Fig 1A and 1B, 0-time point). Degradation was tentatively ascribed to small amounts of contaminating proteases in the μ 2 preparation. Subsequent treatment with chymotrypsin or thermolysin enriched for fragments of similar sizes to those arising during storage (Fig 1A and 1B). When the two fragments from thermolysin digestion were subjected to N-terminal sequencing, the 30K fragment was found to be blocked, but the 50K fragment yielded sequences His-Val-Lys-Arg-Gly and Val-Lys-Arg-Gly-Ala, corresponding to aa 282–287 of T1L μ 2. The first sequence is consistent with cleavage by a chymotrypsin-like protease after Tyr281, while the second sequence is compatible with cleavage by Thermolysin before Val283 (Fig 1C). These results suggested that the 30K and 50K fragments were likely the complementary N- and C-terminal portions of μ 2 (Fig 1C), possibly reflecting two discrete domains of this protein. Based on these findings, we generated a series of new plasmid constructs for the study of protein association and distribution within the cell. These expression plasmids were engineered to express a full-length T1L μ 2 (aa 1–736), its putative 30K portion (aa 1–282), or its putative 50K portion (aa 283–736), in each case fused with EGFP [54] (Fig 1D).

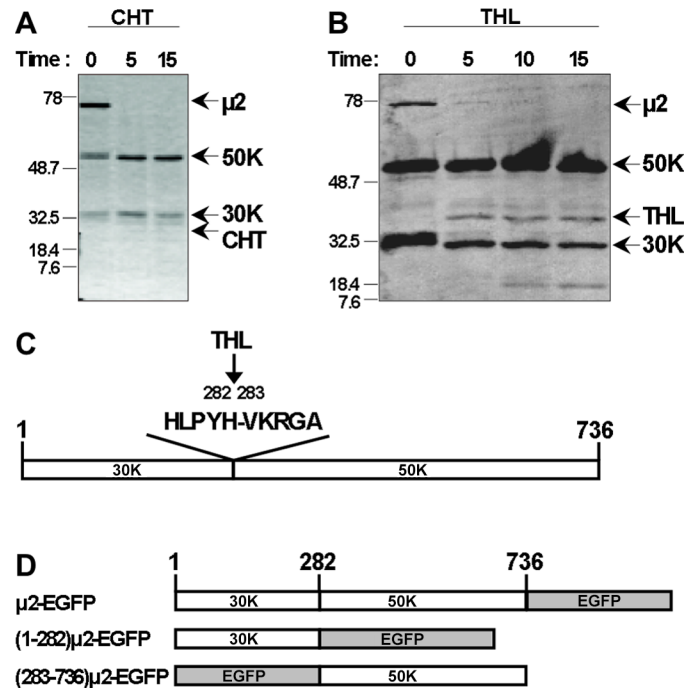


Fig 1. Protease-hypersensitive site in μ 2. Partially purified T1L μ 2 was treated with (A) 40 μ g/ml chymotrypsin (CHT) or (B) 50 μ g/ml thermolysin (THL) at room temperature for the indicated times (in minutes). The proteins were separated in SDS-PAGE, followed by staining in gel with Coomassie blue. Arrows indicate the positions of full-length μ 2, major proteolytic fragments (approximate molecular weights, 50 kDa (50K) and 30 kDa (30K), and the respective protease. The positions of molecular weight markers (kDa) are indicated at left. (C) Schematic representation of μ 2 concluded from the N-terminal sequencing of the major THL-generated fragments. The 30K fragment yielded no N-terminal sequence, consistent with a blocked N-terminus. The 50K fragment yielded sequence Val-Lys-Arg-Gly-Ala (VKRGA), corresponding to μ 2 residues 283–287 and consistent with THL cleavage. (D) Schematic representation of T1L μ 2 full-length, 30K (residues 1–282) and 50K (residues 283–736) fused to an EGFP tag. Not to scale.

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MT-association by an N-terminal portion of EGFP-tagged μ 2

We first tested the μ 2-EGFP fusion proteins for MT-association. After transient expression in CV-1 cells, both μ 2-EGFP and (1–282) μ 2-EGFP exhibited filamentous distributions (Fig 2A), consistent with MT-association as previously shown for non-tagged T1L μ 2 [23]. Additionally, this μ 2 filamentous distribution co-localized with MTs (Fig 2A) or acetylated-MTs (S1A Fig), and was not observed after treatment with the MT-depolymerizing drug nocodazole (S1B Fig). In marked contrast, EGFP-(283–736) μ 2 showed no MT-association and was instead diffusely distributed within the cytoplasm (Fig 2A and S1A Fig). These findings suggest that some portion of μ 2 aa 1–282 is both necessary and sufficient for MT-association in cells (i.e., μ 2 aa 283–736 are dispensable for this activity).

μ NS association by a C-terminal portion of EGFP-tagged μ 2

We next tested the μ 2-EGFP fusion proteins for association with MRV factory-matrix protein μ NS in FLS induced by μ NS [24]. After transient co-expression in CV-1 cells, μ 2-EGFP and EGFP-(283–736) μ 2 were both positive for the μ NS association, whereas (1–282) μ 2-EGFP was negative (Fig 2B). In the case of μ 2-EGFP, the distribution of μ NS was filamentous; reflecting that μ 2-EGFP recruited μ NS to MTs as previously shown for non-tagged μ 2 [24]. In the case of EGFP-(283–736) μ 2, and in contrast with the lack of MT-association, we

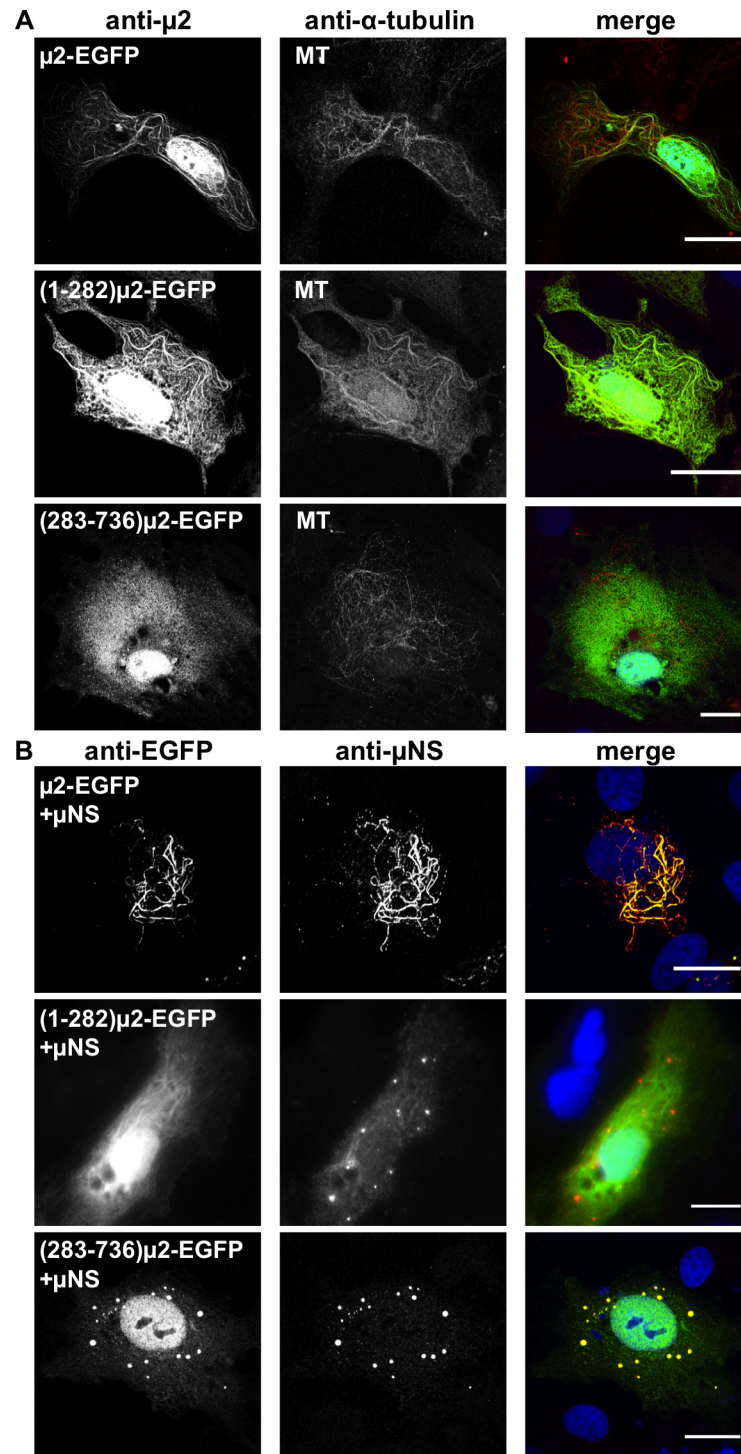


Fig 2. Cellular distribution of T1L μ 2 full-length, aa regions (1–282) and (283–736) fused to EGFP. (A) Confocal immunofluorescence of CV-1 cells expressing T1L μ 2 full-length or both aa regions (1–282) or (283–736) fused to EGFP. At 20 hpt, the cells were methanol fixed and immunostained for the detection of μ 2 (specific polyclonal anti- μ 2 serum, green) (left column) and MTs (mouse mAb anti- α tubulin, red) (middle column). The merged images are shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 20 μ m. (B) Confocal immunofluorescence of CV-1 cells co-expressing T1L μ NS with T1L μ 2 full-length or both aa region (1–282) or (283–736) fused to EGFP. At 20 hpt, the cells were methanol fixed and immunostained for the detection of μ 2 (mouse mAb anti-EGFP, green) (left column) and μ NS (specific polyclonal anti- μ NS serum, red) (middle column). The merged images are shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 20 μ m.

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observed that μ NS was found in globular FLS to which EGFP-(283–736) μ 2 was recruited. As previously shown for non-tagged μ 2 from MRV T3D clones that exhibit limited MT-association at 37°C [24, 30]. In the case of (1–282) μ 2-EGFP, a third distinct pattern was observed since (1–282) μ 2-EGFP exhibited filamentous distribution, reflecting MT-association while μ NS localized separately, to globular FLS, consistent with the lack of μ NS association by (1–282) μ 2-EGFP. All the inspected cells presented an identical pattern when co-expressing of μ 2-EGFP or its deletion mutants with μ NS. These findings suggest that some portion of μ 2 aa 283–736 is both necessary and sufficient for μ NS association in FLS thereby further suggesting that MT-association and μ NS association can be mapped to distinct, respective regions of μ 2.

Additionally, pull-down assay was implemented to assess the interaction of μ 2-EGFP, (1–282) μ 2-EGFP or EGFP-(283–736) μ 2 with μ NS by adding a histidine tag at the EGFP of the μ 2-EGFP derived proteins (S2 Fig). The results show that the three proteins were able to bind μ NS despite the fact that (1–282) μ 2-EGFP-histidine tagged did not localize in FLS, suggesting that a cytosolic soluble portion of these two proteins interact but without localizing in FLS.

Smallest contiguous region of EGFP-tagged μ 2 that mediates MT-association

Additional constructs were evaluated to define the minimal MT-association region of EGFP-tagged μ 2. One set of constructs were designed to express even shorter N-terminal fragments, with progressively longer deletions from the C-terminus of the putative 30K region. Upon transient expression in CV-1 cells, however, even the next-shorter fragment, (1–257) μ 2-EGFP, was negative for MT-association (Fig 3A and 3B). This result suggests that some portion of μ 2 aa 258–282 is critical for MT-association. In this set of experiments, we also tested (1–310) μ 2-EGFP, which was also positive for MT-association and in a similar fraction of transfected cells as μ 2-EGFP and (1–282) μ 2-EGFP (Fig 3A and 3B). Another set of constructs was designed to express EGFP fusions that lacked N-proximal residues of μ 2. Because μ 2 is modified by removal of Met1 and acetylation of Ala2 [55], we retained those residues in these new deletion mutants. By doing so, we ensure the acetylation of Ala in the deletion mutants as assumed by NetAcet prediction method [56]. Interestingly, however, fusions lacking μ 2 aa 3–5 or 3–17, (1–310 Δ 3–5) μ 2-EGFP and (1–310 Δ 3–17) μ 2-EGFP, were negative for MT-association, as observed after transient expression in CV-1 cells (Fig 3A and 3B). These results suggest that an N-proximal region of μ 2 is also important for MT-association. In summary, μ 2 aa 1–282 constitute the smallest contiguous region of EGFP-tagged μ 2 we have shown to be sufficient for MT-association, and sequences near each end of the 1–282 region appear to be essential for this activity.

MT association by HA-tagged μ 2

A caveat, however, is that EGFP weakly dimerizes [57] and might, therefore, display a self-association activity, possibly consequential for MT-association in cells, which is typically provided by a region of μ 2 beyond aa 282 (see below for results consistent with this caveat). To evaluate the μ 2 region that may be sufficient for MT-association in the absence of a self-associating tag like EGFP in the previous experiments, we tested several versions of the μ 2 N-terminal region tagged with influenza virus HA epitope (Fig 4A). Following transient expression in CV-1 cells, (1–282) μ 2-HA and (1–310) μ 2-HA were, surprisingly, negative for MT-association (Fig 4B). In contrast, (1–325) μ 2-HA, (1–338) μ 2-HA, and (1–373) μ 2-HA were all positive, with (1–373) μ 2-HA showing MT association in a similar fraction of transfected cells as μ 2-HA in

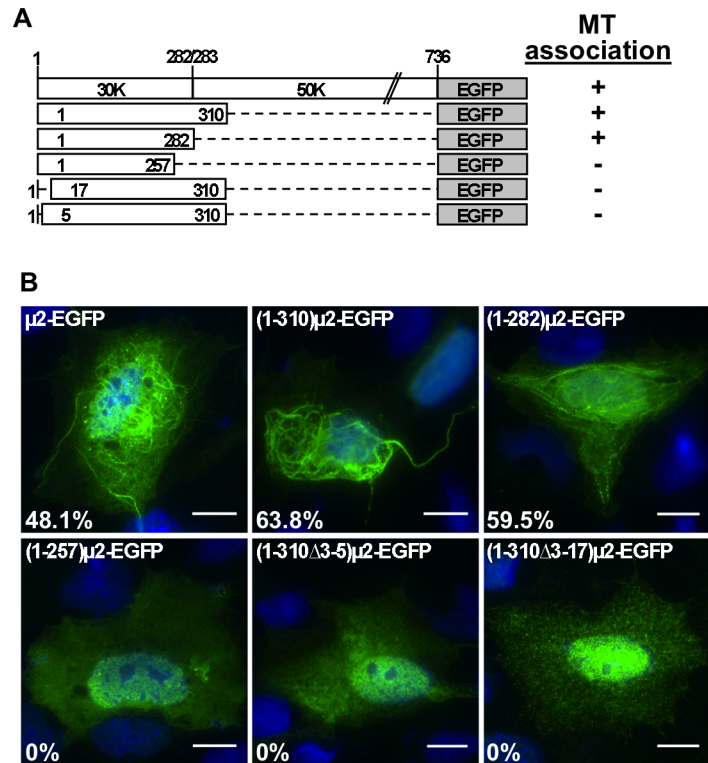


Fig 3. T1L μ2 region 1–282 fused to EGFP is necessary for MT-association. (A) Schematic representation of μ2 N-terminal deletion mutants fused to EGFP constructs (not to scale). Positive (+) or negative (-) for the formation of filamentous phenotype is indicated at right. (B) At 20 hpt, CV-1 cells expressing μ2 full-length or its deletion mutant fused to EGFP, as specified in each panel, were methanol fixed and immunostained for the detection of μ2 (mouse mAb anti-EGFP, green) and nuclei stained with DAPI (blue). The percentage of positive EGFP-tagged protein forming filamentous structures is indicated in each lower left corner. Scale bar is 10 μm.

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the same experiment (Fig 4B). Based on these results, we revised our previous conclusion that some portion of μ2 aa 1–325 is necessary and sufficient for MT-association in cells in the absence of a self-associating protein like EGFP. Moreover, the apparent capacity of EGFP, which oligomerizes in cytosolic localization [58], to substitute for the 283–325 region of μ2 in allowing MT-association by (1–282)μ2-EGFP suggested that residues in the 283–325 region may be involved in μ2 self-association. This self-association may, in turn, be important for MT-association. Residues in the 325–373 region of μ2, which appeared to enhance MT-association by the HA-tagged proteins, might also participate in μ2 self-association. Also notable is that none of the HA-tagged μ2 proteins, except full-length μ2-HA, were positive for μNS association (see below), suggesting that a region of μ2 beyond aa 373 is required for the μNS association.

Point mutations in μ2 that abrogate MT-association

The putative self-association region of μ2, residues 283–325, contains four Val-Asp-Val repeats in residues 290–307 (Fig 5A). We hypothesized that these repeats might be involved in a mechanism encompassing 283–325 region that contributes to MT-association. To test this hypothesis, we engineered single Ala substitutions for residues Asp291, Asp296, Asp299, and Asp306 in the full-length μ2-HA. When transiently expressed in CV-1 cells, both μ2(D296A) and μ2(D306A) show a cytosolic filamentous distribution that is consistent with MT-association.

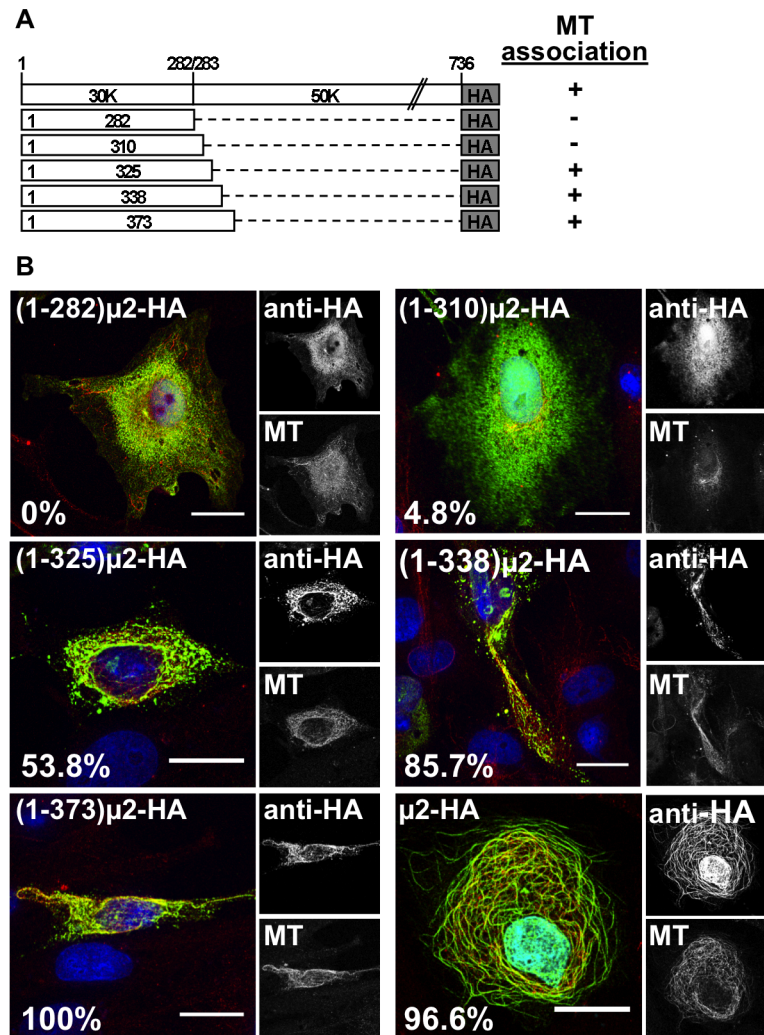


Fig 4. μ 2 region 1 to 373 fused to HA tag is necessary for MT-association. (A) Schematic representation of T1L μ 2 N-terminal deletion mutants fused to an HA tag (not to scale). Positive (+) or negative (-) MT-association phenotype is indicated at right. (B) Confocal immunofluorescence of CV-1 cells expressing μ 2 or its deletion mutants with HA tag. At 20 hpt, cells were methanol fixed and immunostained for the detection of μ 2 or its deletion mutants (mouse mAb anti-HA, green) (right upper panel) and MTs (specific polyclonal anti-alpha tubulin serum, red) (right lower panel). A Merged image is shown at the left of each panel. Nuclei are stained with DAPI (blue). Scale bar is 20 μ m. The percentage of positive cells HA-tagged proteins associating to MTs is indicated at the left bottom of each merged image.

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Remarkably, when expressing μ 2(D291A) or μ 2(D299A), a cytosolic homogenous distribution was observed with a negative co-localization to the MT-network (Fig 5B). Notably, all four of these mutants remained positive for association with MRV factory-matrix protein μ NS (S3 Fig and Table 1). Furthermore, other μ 2 mutations, Y293F, R312K, S315A, R316A, and R316K, were also examined in the setting of full-length μ 2, and all expressed proteins showed filamentous distribution and remained positive for association with both MTs (Fig 5B and Table 1) and μ NS (S3 Fig). In the case of mutant μ 2(Y293L), it was negative for MT-association (Fig 5B) but remained positive for association with the μ NS association in globular FLS (S3 Fig). Thus, only three single-site substitutions within the 283–325 region of μ 2, D291A, Y293L, and D299A, were found to abrogate MT-association, and none of these affected μ NS association (Table 1).

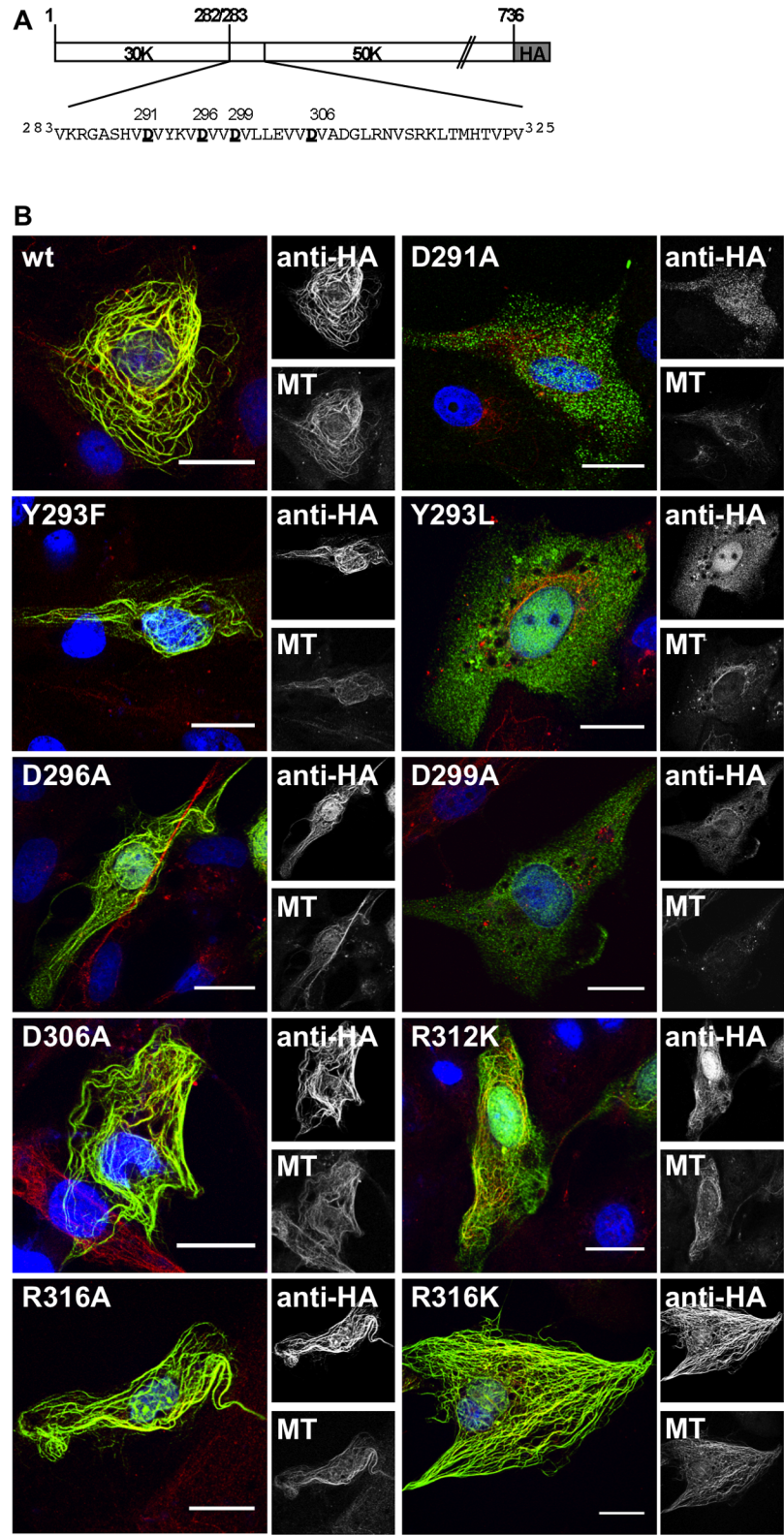


Fig 5. Point mutations in a repetitive motif in μ 2 region, 283 to 325, abrogate MT-association. (A) Schematic representation of the amino acid sequence in T1L μ 2 region 283 to 325. The Asp in each Val-Asp-Val motif is underlined. In the scheme, the μ 2 is fused at the C-terminus to an HA tag. (B) Confocal immunofluorescence of CV-1 cells expressing μ 2-HA wild type or containing the specified point mutation. At

20 hpt, cells were methanol fixed and immunostained for detection of μ 2 (mouse mAb anti-HA, green) (right upper panel) and MTs (specific polyclonal anti-alpha tubulin serum, red)(right lower panel). A Merged image is shown at the left of each panel. Nuclei are stained with DAPI (blue). Scale bar is 20 μ m.

<https://doi.org/10.1371/journal.pone.0184356.g005>

μ 2 self-association in a cytoplasmic platform assay and point mutations that abrogate it

We next tested directly whether μ 2 residues 283–325 are involved in μ 2 self-association. Previous studies have validated and shown the usefulness of (471–721) μ NS as a platform for probing protein-protein associations in large cytoplasmic FLS [47–50, 59]. For this purpose, we fused the μ 2 residues from region 283–325 to both the N-terminus of EGFP as well as to an mCherry-(471–721) μ NS cytosolic platform. As observed in Fig 6A, when co-expressing EGFP with mCherry-(471–721) μ NS (Fig 6A, first row) or both carrying a (283–325) μ 2 region independently were unable to co-localize in the cytosolic platform (Fig 6A, second and third rows). However, when co-expressing (283–325) μ 2-EGFP with (283–325) μ 2-mCherry-(471–721) μ NS (Fig 6A, fourth row), a positive cytosolic FLS platform co-localization was observed. Taken together, μ 2 aa residues 283–325 resulted being sufficient for a self-association activity.

As mentioned above, point mutations in D291A, Y293L, and D299A of μ 2 were sufficient to abrogate the MT-association (Fig 5), suggesting that one possibility is that these residues affect μ 2 self-association. Under those circumstances, one would expect that mutations in these residues would also disrupt association by the 283–325 region in the platform assay. Indeed, when these mutations were tested, all three abrogated co-localization in the platform assay when present in either one of the co-expression partners or both (Fig 6B and S4 Fig).

These results provide further evidence for the involvement of the 283–325 region in a μ 2 self-association activity. Notably, the point mutations D291A, Y293L, and D299A are involved in the abrogation of both MT-association and μ 2-self-association.

Table 1. Summary of associations by μ 2-HA and specified point mutations.

Plasmid-expressed μ 2-HA protein ^a	MT-association ^b	μ NS association ^c	μ 2 self-association ^d
WT	+	+, F	+
D291A	-	+, G	-
Y293F	+	+, F	NT
Y293L	-	+, G	-
D296A	+	+, F	NT
D299A	-	+, G	-
D306A	+	+, F	NT
R312K	+	+, F	NT
S315A	+	+, F	NT
R316K	+	+, F	NT
R316A	+	+, F	NT

^a μ 2-HA containing wild-type (WT) μ 2 or the specified μ 2 point mutation was expressed in plasmid-transfected CV-1 cells.

^b Ascertained by co-immunostaining with for μ 2 with a specific mAb anti-HA and MT with anti-tubulin-Alexa 488.

^c Ascertained by co-expression of μ NS and μ 2-HA, followed by immunostaining with μ NS-specific polyclonal antibodies and HA-specific mAb. Morphology of the FLS is also indicated: F, filamentous; G, globular.

^d Ascertained by (283–325) μ 2 region fused to cytoplasmic platform assay as described in the text.

NT, not tested.

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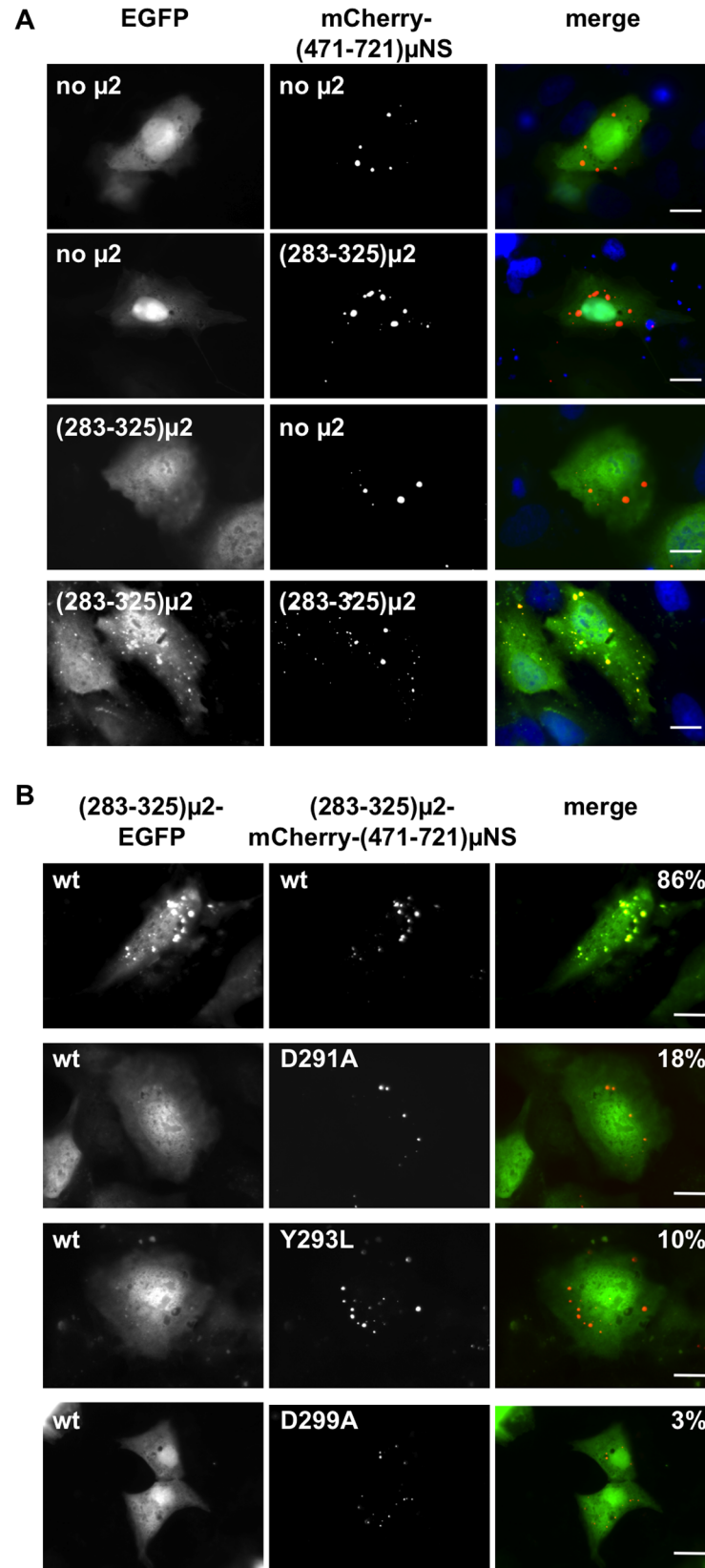


Fig 6. The amino acids D291, Y293 and D299 present in μ 2 region from 283 to 325 are required for μ 2 self-association. An *in vivo* protein interaction platform was performed, in which EGFP-tagged protein is "fish" and mCherry-tagged protein is "bait." (A) At 20 hpt, CV-1 cells co-expressing either EGFP (first and second rows) or (283–325) μ 2-EGFP (third and fourth rows) with either mCherry-(471–721) μ NS (first and third rows) or (283–325) μ 2-mCherry-(471–721) μ NS (second and fourth rows) were fixed and analyzed by fluorescence microscopy. A merged image is shown in the right column. Scale bar is 10 μ m. (B) At 20 hpt, CV-1 cells co-expressing the "fish" (283–325) μ 2-EGFP with the "bait" (283–325) μ 2-mCherry-(471–721) μ NS, respectively. (283–325) μ 2-mCherry-(471–721) μ NS containing μ 2 wild-type or its individual point mutations are specified in the left top corner of each picture from the middle column. Cells were fixed and visualized by fluorescence microscopy. A merged picture is shown in the right column (EGFP, green; mCherry, red and nuclei stained with DAPI, blue). Scale bar is 10 μ m.

<https://doi.org/10.1371/journal.pone.0184356.g006>

μ 2 self-association involving the 283–325 region is important for MT-association

We took further advantage of the three point mutations D291A, Y293L, and D299A to address whether the μ 2 self-association involving the 283–325 region is only important for μ 2 association with MTs or whether the μ 2 self-association involving other regions might be sufficient. To address this question, we co-expressed μ 2-EGFP, as a known MT-associating protein (Fig 2), with μ 2-HA, either with or without the point mutations in the latter. Although these mutations abrogate association with MTs by μ 2-HA when expressed alone (Fig 5), the mutant proteins might associate with MTs in this co-expression setting if they can form functional hetero-oligomers with μ 2-EGFP. On the other hand, if the self-association mediated by the 283–325 region is important for MT-association or is the only means of μ 2 self-association, then these mutant proteins would remain negative for MT association even in the presence of μ 2-EGFP.

To begin these experiments, μ 2-EGFP and μ 2-HA were co-expressed and found to co-localize on MTs (Fig 7A, first row), as expected from our previous results. When μ 2-EGFP was co-expressed with μ 2(D291A)-HA or μ 2(Y293L)-HA, however, there was little or no colocalization, and the HA-tagged mutants remained diffusely distributed (Fig 7A, second and third rows). Interestingly, when μ 2-EGFP was co-expressed with μ 2(D299A)-HA, only weak colocalization on MTs was observed, but in a majority of the cells (Fig 7A, fourth row). When μ 2-EGFP was co-expressed with μ 2(R316A)-HA, as a control for a mutant that retains MT-association activity on its own (Fig 5 and Table 1), strong co-localization on MTs was again observed, comparable to that seen with μ 2-HA (Fig 7). These results provide further evidence that μ 2 residues Asp291 and Tyr293 are important for self-association involving the 283–325 region of μ 2. Residue Asp299 appears to be somewhat less important in this respect since the D299A mutation allowed consistently weak colocalization in this assay.

Interestingly, these results indicate that μ 2 self-association involving the 283–325 region is specifically important for MT-association. Thus, in the absence self-association ability, the mutation-containing μ 2-HA proteins not only failed to associate with MTs on their own (Fig 5) but also failed to be recruited to MTs by μ 2 *in trans* (Fig 7).

μ 2 region necessary and sufficient for the association with matrix protein μ NS in FLS

As shown above, when the 50K region of μ 2 was fused to EGFP it was unable to associate to MTs, but instead, it associates to μ NS by co-localizing in FLS (Fig 2). To investigate which is the minimal region of μ 2 necessary and sufficient for the association to μ NS in FLS, we sequentially deleted μ 2 from its N- and C-terminus, fusing an HA-tag at the C-terminal of each deletion mutant (Fig 8A). These μ 2 deletion mutants were analyzed for their capacity to associate with μ NS in FLS. As expected, μ 2 full-length-HA and deletion mutant (283–736) μ 2-HA

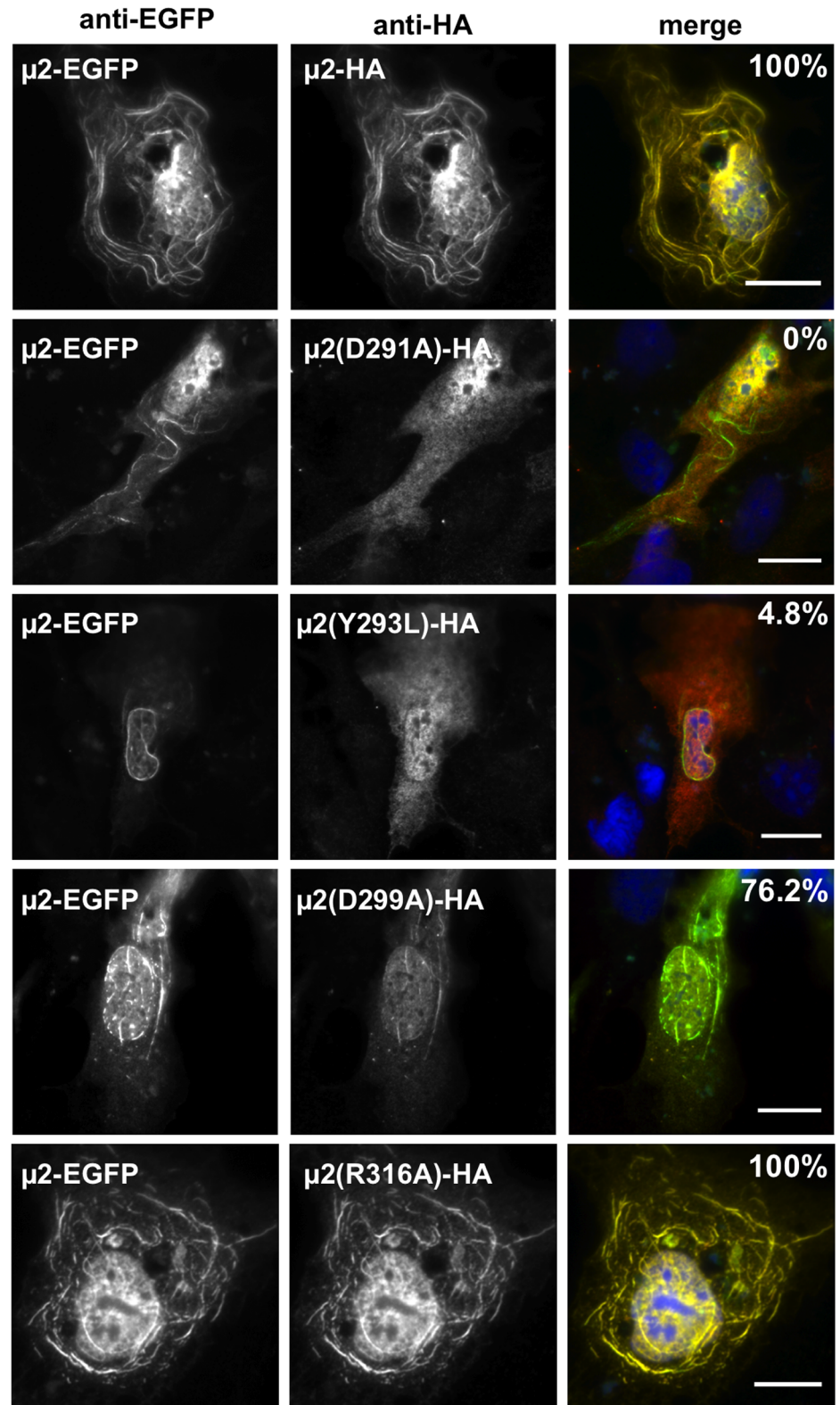


Fig 7. Amino acid residues Asp 291, Tyr 293 and Asp 299 abrogate μ 2 self-association. (A) Immunofluorescence of CV-1 cells co-expressing both μ 2 wild type fused to EGFP and μ 2 wild type or containing the specified point mutation tagged to HA. At 20 hpt, cells were methanol fixed and immunostained for the visualization of μ 2-EGFP (specific anti-EGFP serum, green) (left column) and μ 2-HA and individual

point mutations (mAb anti-HA, red)(middle column). Merged images are shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 10 μ m. The percentage of positive cells co-expressing μ 2-HA and μ 2-EGFP, which form filamentous structures when associating is indicated in the top-right merged images.

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associate with μ NS when co-expressed, forming filamentous and globular FLS respectively (Fig 8B, first and second rows). As anticipated, the 30K region of the μ 2 construct (1–282) μ 2-HA could not localize in globular μ NS FLS (Fig 8). We then systematically deleted the N-terminus of μ 2, checking for its ability for μ NS-FLS localization. Our data show that making a deletion from residue 290 the capacity to localize in globular μ NS-FLS is retained, but this ability is lost when deleting up to residue 298 (Fig 8 and S5 Fig). Next, we engineered μ 2 constructions with sequential deletions from its C-terminus. From these constructions, our results indicate that the μ 2 up to residue 453 was necessary to retain the capacity to localize in globular μ NS-FLS (Fig 8). We then tested a shorter μ 2 construction, including residues 290 to 453, resulted in a positive co-localization to μ NS-FLS. This outcome was, in fact, the minimal region necessary and sufficient of μ 2 required to associate with μ NS in FLS, since shorter versions, like (290–437) μ 2-HA, were unable to localize in μ NS-FLS remaining diffuse in the cytosol (Fig 8 and S5 Fig). Notably, the construction 290 to 453 also encompasses the μ 2 self-association region.

Discussion

The lack of an X-ray crystal structure for the MRV μ 2 protein, the only MRV structural protein for which this restriction remains [4, 8, 60–62], has made it difficult to define the regions involved in its activities, which include RNA binding [21]; hydrolysis of the γ -phosphate from NTPs and RNA 5'-termini [13, 15]; and associations with λ 3 [13], μ NS [24], and MTs [13, 23]. A better understanding of these activities should shed light on the roles of μ 2 in the MRV life cycle and its effects on cells, e.g., as a critical component of the transcriptase and replicase complexes [16, 18, 63] and viral factories [14, 22, 23, 29, 30]. Of particular interest is the basis for genetic influences of M1/ μ 2 on MRV growth in different cells and in MRV growth and virulence in animals [33, 35, 39, 40, 41, 42].

In this study, we made a dissection of several specific activities of μ 2. We began by exploring its association with MTs in cells and, through those studies, we were led to investigate a newly identified activity, i.e., the self-association involving the aa 290–325 region. The new evidence for μ 2 self-association is not surprising. Preliminary results (X. Lu, J.K., M.L.N., and S.C. Harrison; also see reference [13]) have suggested that μ 2 takes the form of a dimer or other small oligomer upon purification. Moreover, stoichiometric determinations from protein gels of MRV particles have suggested that there are ~20 copies of μ 2 per virion or core [5], consistent with two copies of μ 2 for either each of the ten genome segments (20 copies) or each of the twelve icosahedral fivefold vertices (24 copies). Evidence for localization of μ 2 to the vertices has come from a reduction in the intensity of the μ 2 protein band in combination with a decrease in the density of inwardly projecting transcriptase complexes near the fivefold axes of protease-treated, genome-deficient particles [63]. The fivefold axes are where λ 3 (the viral RdRp) is located [64], so the μ 2- λ 3 interaction shown with purified proteins [13] is consistent with this location of μ 2. Whether self-association is essential for the functions of μ 2 in cores (NTPase, RTPase, putative RNA helicase, a putative cofactor for the viral RdRp in transcriptase and replicase complexes) [13, 15, 16, 18] remains yet to be elucidated. The self-association activity was mapped by deletion mutations to the μ 2 residues 283–325, with point mutations assigning specific importance to Asp291, Tyr293, and Asp299. Notably, the μ 2 homolog protein from avian orthoreovirus, μ A, shows a strong degree of sequence conservation with μ 2

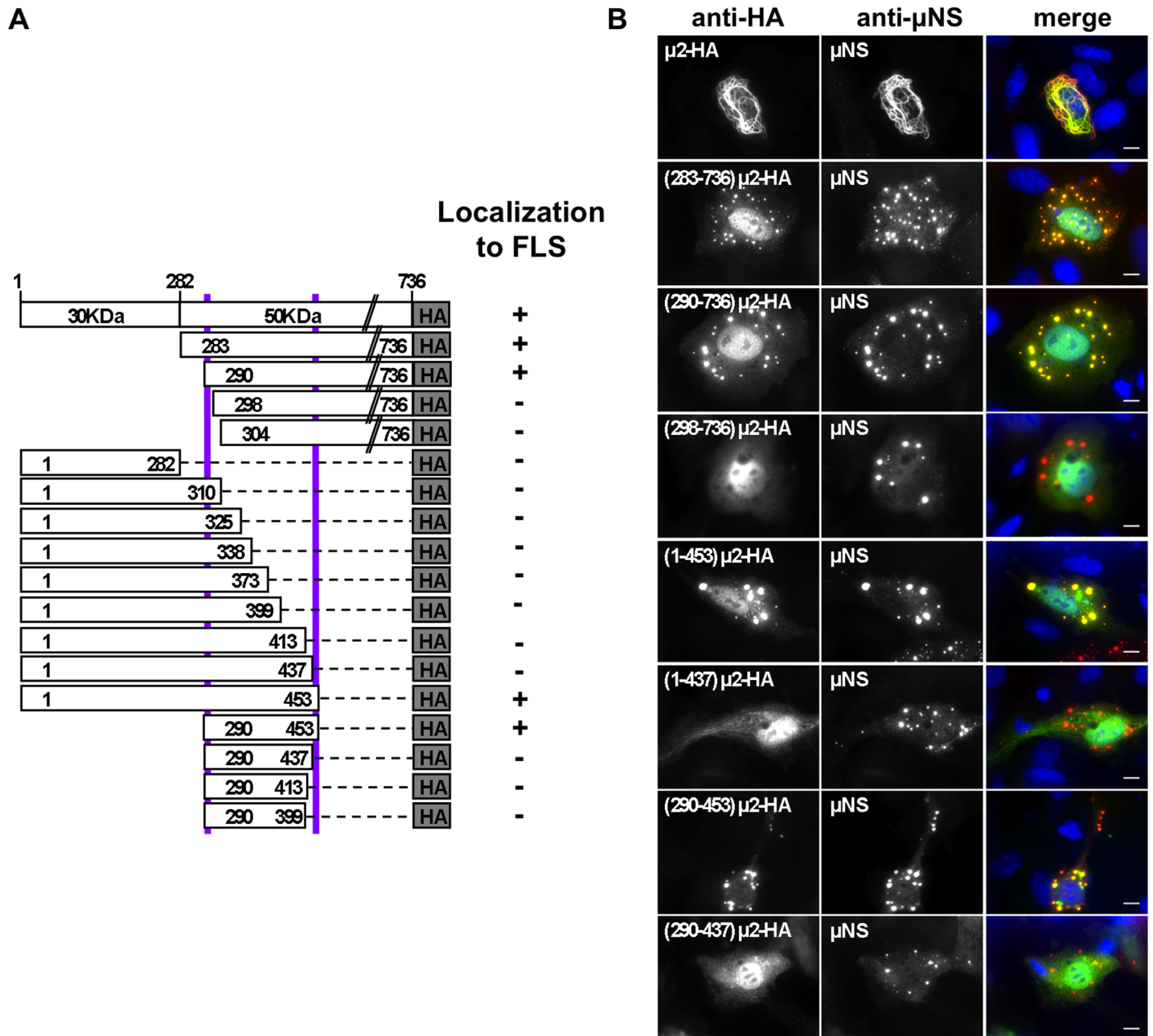


Fig 8. $\mu 2$ region necessary and sufficient for μ NS association in FLS. (A) Schematic representation of T1L $\mu 2$ deletion mutants fused to an HA tag (not to scale). Positive (+) or negative (-) co-localization to μ NS-FLS phenotype is indicated at right. (B) Most representative immunofluorescence of CV-1 cells co-expressing the indicated $\mu 2$ deletion mutant HA-tagged along with T1L μ NS. At 20 hpt, cells were fixed and immunostained for detection of $\mu 2$ (mouse mAb anti-HA, green) (left column) and μ NS (specific anti- μ NS serum, red)(middle column). A merged image is shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 10 μ m.

<https://doi.org/10.1371/journal.pone.0184356.g008>

residues 290–307 (Fig 9A), suggesting that self-association may be common to many or all members of the genus *Orthoreovirus*. In the case of VP5, the $\mu 2$ homolog protein from aquareoviruses, there is sequence conservation with $\mu 2$ residues 290–307 as well (Fig 9A), suggesting that members of the related genus *Aquareovirus* may also share this self-association activity. Of notice is the fact that self-association has not yet been experimentally demonstrated

A

			* * *																	
MRV T1L	290	V	D	V	Y	K	V	D	V	V	D	V	L	F	E	V	V	D	V	307
MRV T2J	290	V	D	V	Y	R	V	D	V	V	D	V	L	F	E	V	V	D	I	307
MRV T3D	290	V	D	V	Y	K	V	D	V	V	D	M	L	F	E	V	V	D	V	307
MRV T1C29	290	V	D	V	Y	K	V	D	V	V	D	V	L	F	E	I	V	D	V	307
MRV T1N84	290	V	D	V	Y	K	V	D	A	V	D	V	L	F	E	V	V	D	V	307
MRV T2S59	290	V	D	V	Y	R	V	D	V	V	D	V	L	L	E	V	V	D	V	307
MRV T3C44	290	V	D	V	Y	K	V	D	V	V	D	V	L	F	E	I	V	D	V	307
ARV 138	283	I	N	T	S	I	I	Q	V	I	D	L	R	L	Q	C	R	H	S	300
ARV 176	283	I	N	T	S	I	I	Q	V	V	D	L	K	L	Q	C	R	H	S	300
ARV S1133	283	I	N	T	S	I	I	Q	V	V	D	L	K	L	Q	C	R	H	S	300
ARV 918	283	I	N	T	S	I	I	Q	V	V	D	L	R	L	Q	C	R	H	S	300
ARV S14	283	I	N	T	S	I	I	Q	V	I	D	L	R	L	Q	C	R	H	S	300

putative motif: ØdbhXØdvvDØXØdXXXX

AqRV GCRV	282	F	D	F	S	A	L	P	V	V	D	I	C	L	L	E	S	E	299	
AqRV GSRV	282	F	D	F	S	A	L	P	V	V	D	I	C	L	F	E	S	E	299	
AqRV GIRV	282	H	N	R	H	H	V	P	V	C	D	L	Q	C	A	L	S	P	E	299

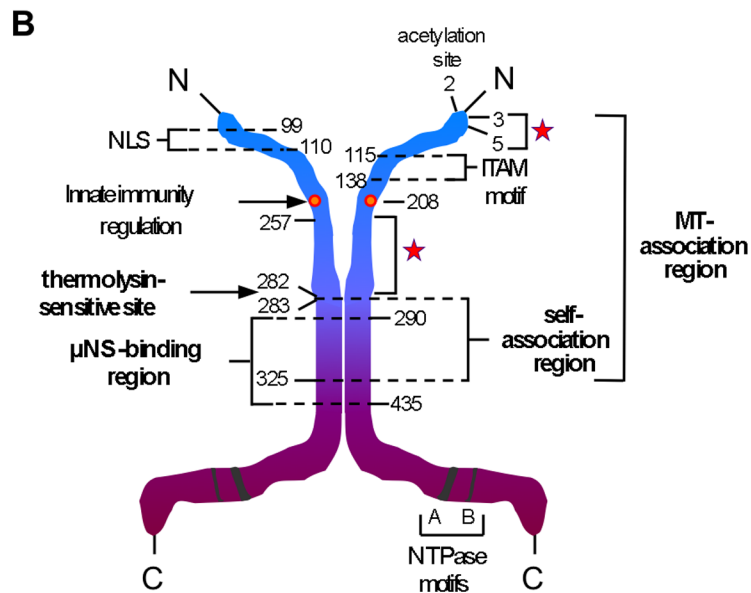


Fig 9. Self-association motifs sequence and model for μ2 binding regions. (A) T-Coffee alignment of μ2-homolog proteins in the 290–307 region of μ2. GenBank accession numbers are listed in Materials and Methods. T2J, Type 2 Jones; T1C29, Type 1 Clone 29; T1N84, Type 1 Netherlands 1984; T2S59, Type 2 Simian Virus 59; T3C44, Type 3 clone 44; GCRV, Grass carp reovirus; GSRV, Golden shiner reovirus; GIRV, Golden ide reovirus; see text for other abbreviations. Members of the genus *Orthoreovirus*, MRVs and avian reoviruses (ARVs), are listed above the putative sequence motif defined by the MRV and ARV sequences. Members of the related genus *Aquareovirus*, aquareoviruses (AqRVs), are listed below the motif. In the motif, Ø means hydrophobic residue (A, F, I, L, M, V) (letters in blue-green), d means acid or amide residues (D, E, N, Q) (letters in orange), b means b-branching residues (T, V) (letters in purple), h means hydroxylated residues (S, Y) (letters in yellow-green), X means variable residues (letters in black), v means mostly conserved Val residue (letters in blue-green), and D means conserved Asp residue (letters in orange). Shading identifies residues that vary from the consensus within each group (MRVs, ARVs, and AqRVs). Residues shown by point mutagenesis to be important for μ2 self-association and microtubule association in this study are indicated above by (*). (B) Summary diagram of MRV μ2. The 736-aa μ2 protein is represented as a dimer, having separate N- and C-terminal domains and a central region of self-association. The putative N-terminal domain (residues 1–282), acetylated at Ala2, is shown in blue, and the putative C-terminal domain (residues 283–736) is shown in purple. The unique protease-hypersensitive region is found between these two domains, N-terminally abutting the self-association region. The self-association region might represent a

separate, central domain. Residues 1–325 were shown to be the minimal contiguous region for MT-association in cells, as labeled. Three smaller regions were determined to be important for MT-association. Two of these regions, residues 3–5 and 257–282, are each indicated by a red star; the third, residues 283–325, is also necessary for μ 2 self-association, as labeled. Mutation P208S, previously related to loss of MT-association and innate immunity regulation [23], is indicated by an orange dot. Residues 99–110 [14] and 115–118 [32] are described as sufficient for μ 2 nuclear localization (NLS) and as related to immunoreceptor tyrosine-based activation motif (ITAM) signaling cascade, respectively. The two previously identified NTPase/RTPase motifs, A (residues 410–420) and B (residues 446–449) [15, 70], are also indicated. The association region for MRV factory-matrix protein μ NS has been mapped between residues 290 and 399.

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for these μ 2 homologs of MRV relatives. To our knowledge, among ortho- and aquareoviruses, only MRVs have so far been shown to have MT-anchored viral factories [23, 26, 27, 29, 65–67], suggesting that any self-association by μ 2 homologs μ A and VP5 may have evolved and been maintained for reasons other than for MT-association, such as for the NTPase/RTPase activity or related functions [68, 69].

In this study, only the 283–325 region was shown to be sufficient for μ 2 self-association, as reflected in the model diagram (Fig 9B). But might other regions of μ 2 be in and/or sufficient for this activity as well? Our experiments to date have not addressed this question, although we cannot exclude that other additional μ 2 activities require an oligomeric conformation, as is the case for nuclear transport [14] or regulation of host innate immune response mediated through an ITAM motif [32]. Since association with μ NS by any of the three point mutations that abrogate association with both MTs and self (Table 1), self-association involving the 283–325 region of μ 2 appears not to be essential for the μ NS association.

The new evidence that an N-terminal region of μ 2 is necessary and sufficient for MT association is also not surprising. In particular, previous results have shown that point mutation P208S reduces MT-association, such as in our lab's version of MRV strain T3D [23, 29, 30]. This mutation appears to promote temperature-dependent misfolding of μ 2 protein, with a subsequent increase in aggregation and polyubiquitination, along with reduced MT-association [30]. Lowered expression temperature (31 vs. 37°C) partially restores MT-association activity and reduces aggregation and polyubiquitination of P208S-containing μ 2 [30]. Perhaps this folding defect is limited to the N-terminal, MT-binding domain of μ 2 (Fig 9B), being other parts and functions of μ 2 remaining intact. An important question is how self-association involving the 283–325 region of μ 2 can be substantial for MT-association. In this case, the self-association involving some portion of residues 283–325 might contribute to forming a basal oligomer of μ 2, such as a dimer as modeled in Fig 9B. This self-association would be important for stabilizing the MT-association, perhaps by allowing interaction of each μ 2 oligomer to bind with MTs bundles. The self-association involving some portion of residues 283–325 might instead also represent a higher-order association between basal oligomers of μ 2 that is relevant for stabilizing the association of μ 2 oligomers playing a role in stabilizing MT-association, either within the same MT or MT-bundles. The observation that a weakly self-associating tag, specifically EGFP, can substitute for the 283–325 region of μ 2 in promoting MT-association (Figs 2 and 4) is consistent with either of the proposed explanations.

There are many different kinds of cellular MT-associated proteins (MAPs), including the MAP1 and MAP2/Tau families (reviewed in [71] and [72]). These various families have different sequence requirements for binding to MTs. Many MAPs have hydrophobic regions involved in MT association, and the 164–282 region of μ 2 is also relatively hydrophobic [29]. Some MAPs undergo post-transcriptional modification to enhance their binding to MTs, such as phosphorylation of MAP1B (reviewed in reference [73]). Preliminary data, however, indicate that μ 2 is not phosphorylated, suggesting that this kind of signal transduction not be involved in the association between μ 2 and MTs.

We also dissected the association of μ 2 in FLS with MRV factory-matrix protein μ NS, defined as the μ 2 region 290–453. Notably, this region overlaps with part of the conserved μ 2 self-association domain (aa 290–307) suggesting a tight regulation regarding the viral factory morphologies. As discussed above, the μ NS association to μ 2 is not impaired by disruptive self-association point mutations (D291A, Y293L, and D299A). On the same lines, previous evidence has shown that the morphology of FLS obtained after co-expression of μ 2 and μ NS is dependent upon the expression ratios of μ 2 and μ NS, with higher μ 2 or μ NS ratios forming filamentous or globular FLS, respectively [24]. This pattern can be observed during viral factories dynamics from the filamentous strain (like MRV T1L), in which at early and late times post-infection factories are seen as globular or filamentous, respectively. This effect also correlates with higher expression levels of μ NS at early times post-infection and with increasing expression levels of μ 2 at later times post-infection. In our model, we propose that μ NS binds μ 2 at region 290–453 at early times post-infection constituting globular viral factories and then at later times post-infection, the increased amounts of μ 2 leads to its oligomerization allowing the MT binding and stabilization, which in turn form filamentous viral factories. Based on these data, we predict that over-expressing a μ 2 dominant-negative carrying a point mutation in D291A, Y293L or D299A will lead to the generation of globular viral factories filamentous strain. Our data also suggest that a soluble portion of both μ 2 and μ NS can interact in the cytosol in the absence of FLS. This result is not surprising since it has been described for other members of the *Reoviridae* family, such as the association of the rotavirus proteins NSP2 and NSP5 in the soluble cytosolic portion, independently of viroplasm assembly [74]. The implications of these soluble interactions are not addressed in this study.

By associating with and stabilizing MTs, as well as anchoring the viral factories to them in the case of most MRV strains, μ 2 has the potential for many different types of effects on host cells and tissues. To name just a few broad examples, μ 2 could affect functions of MTs in the transport of vacuoles and other cargo, maintenance, and organization of cellular organelles, cell polarity, and adherence, cell motility, ciliary function, mitosis, and/or cytokinesis. Thus, learning more about μ 2-MT associations and secondary effects may be key to understanding the roles of μ 2 in growth and virulence in infected animals.

Supporting information

S1 Fig. (A) Confocal immunofluorescence of CV-1 cells expressing EGFP-tagged full-length, aa regions 1–282 or 283–736 from T1L μ 2. At 20 hpt, cells were methanol fixed and immunostained for the detection of μ 2 (specific anti-EGFP serum, green) (left column) and acetylated-MTs (mAb anti-acetylated alpha tubulin, red)(middle column). A merged image is shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 20 μ m. (B) Immunofluorescence of CV-1 cells expressing EGFP-tagged full-length T1L μ 2 or (1–282) μ 2. At 23 hpt, cells were treated for 1 hour with 10 μ M nocodazole. Afterward, cells were methanol fixed and immunostained for the detection of μ 2 (specific anti-EGFP serum, green)(left column) and MTs (mAbs anti-alpha tubulin, red). The merged images are shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 20 μ m.

(TIF)

S2 Fig. Pull down assay of CV-1 lysates co-expressing histidine tagged μ 2-EGFP deletions and μ NS at a transfection ratio of 2:1. (A) At 15 hpt, cell cultures images were acquired at the fluorescent microscope. Scale bar is 20 μ m. (B) Immunoblotting of pulled down samples (cellular extract (ce), flow through (ft) and elution (el)) from nickel resin. Previous to lysis, cells were DSP cross-linked. The membranes were incubated with anti-EGFP, and anti- μ NS for the detection of μ 2-EGFP-H₆ derived proteins (upper panel) and μ NS (lower panel), respectively.

The red dots show the monomeric isoform of μ 2-EGFP-H₆ or its derived deleted proteins. (TIF)

S3 Fig. Immunofluorescence of CV-1 cells co-expressing T1L μ 2-HA tag or the indicated point mutations with T1L μ NS. At 20 hpt, cells were methanol fixed and immunostained for the detection of μ 2 (mAb anti-HA, green) (left column) and μ NS (specific anti- μ NS serum, red)(middle column). The merged images are shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 10 μ m. (TIF)

S4 Fig. At 20 hpt, CV-1 cells co-expressing the "fish" (283–325) μ 2-EGFP along with both (283–325) μ 2-mCherry-(471–721) μ NS (left panel) or mCherry-(471–721) μ NS (right panel) as "baits," containing wild type- μ 2 or its point mutations as labeled, respectively. Cells were fixed and directly visualized by fluorescence microscopy. A merged image is shown in each right column (EGFP (green), green; mCherry (red) Scale bar is 10 μ m. (TIF)

S5 Fig. Immunofluorescence of CV-1 cells co-expressing T1L μ 2-HA tag or deletion mutants with T1L μ NS. At 20 hpt, cells were methanol fixed and immunostained for the detection of μ 2 (mAb anti-HA, green) (left column) and μ NS (specific anti- μ NS serum, red) (middle column). The merged images are shown in the right column. Nuclei are stained with DAPI (blue). Scale bar is 10 μ m. (TIF)

S1 Table. Primers for the μ 2 deletion mutants and for the cytoplasmic platform segments constructions. (DOCX)

S2 Table. Oligonucleotides used to introduce point mutations in μ 2-HA. (DOCX)

S1 File. Supporting materials and methods. Plasmid construction. (DOCX)

Acknowledgments

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