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Supplemental Information

Coronaviruses Hijack the LC3-I-Positive EDEMosomes, ER-Derived Vesicles Exporting Short-Lived ERAD Regulators, for Replication

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Supplemental Experimental Procedures

Cell culture, viruses

HEK293, HeLa, HeLa-CEACAM1a [HeLa cells stably expressing the MHV receptor mCEACAM1a, (Verheije et al., 2008)], murine LR7, shLUC HEK293, shEDEM1/shOS-9 HEK293 cells and, *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ MEF [a gift of M. Komatsu, The Tokyo Metropolitan Institute Medical Science, (Komatsu et al., 2005)] were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 units/ml of penicillin and 0.1 mg/ml of streptomycin (all from Invitrogen). The shLUC and shEDEM1/shOS-9 HEK293 cell lines stably silenced for luciferase and both splice variants of OS-9 plus EDEM1, respectively, were generated as described (Bernasconi et al., 2008). Targeting sequences were GCTGCCTACCTGGAGATTCAG for OS-9 and ACAGATAGTCTCTTAGGTTAT for EDEM1. The HEK293 cells stably transfected with a plasmid expressing GFP-LC3 (Kochl et al., 2006), kindly provided by S. Tooze (Cancer Research UK, London), were grown in the same medium supplemented with 0.5 mg/ml of G418 (Invitrogen). HeLa cells were transiently transfected with the plasmid expressing GFP-LC3 (a gift of K. Kirkegaard, Stanford University) using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol.

LR7 mouse cells were used to propagate the wild type and recombinant MHVs (based on the A59 strain). The recombinant viruses expressing the firefly luciferase gene (MHV-2aFLS), nsp2-GFP (MHV-nsp2GFP) or the spike protein that gives the virus an extended host range (MHV-Srec) have been described elsewhere (de Haan et al., 2005; de Haan et al., 2003; Verheije et al., 2008).

Plasmid construction

LC3B was amplified by PCR from cDNA (rzpd) using appropriate primers and cloned into both the pDest-520 vectors (Clontech) generating the pLC3B-HA plasmids. The 3' primer used to create the C-terminally tagged version of LC3B was designed such that it mutates the penultimate glycine of LC3B into an alanine so that the resulting protein cannot be converted into LC3-II by proteolytic cleavage and subsequent lipidation. In addition, silent mutations were introduced into the *LC3B* nucleotide sequence in order to have the LC3B-HA protein fusion not knockdown by the employed siRNA duplexes.

Statistical analysis

The number of punctate LC3 or GFP-LC3 fluorescent dots was counted in the central section of each cell. Proportions of LC3 or GFP-LC3 puncta co-localizing with nsp2/nsp3 were fitted with a generalized linear model in R version 2.8.1 (R Development Core Team 2009), using the glm() procedure and the family=quasibinomial option to correct for overdispersion. Differences between LC3 and GFP-LC3 were assessed with the t-test.

References

de Haan, C.A., Li, Z., te Lintelo, E., Bosch, B.J., Haijema, B.J., and Rottier, P.J. (2005). Murine coronavirus with an extended host range uses heparan sulfate as an entry receptor. J Virol *79*, 14451-14456.

de Haan, C.A., van Genne, L., Stoop, J.N., Volders, H., and Rottier, P.J. (2003). Coronaviruses as vectors: position dependence of foreign gene expression. J Virol *77*, 11312-11323.

Kochl, R., Hu, X.W., Chan, E.Y., and Tooze, S.A. (2006). Microtubules facilitate autophagosome formation and fusion of autophagosomes with endosomes. Traffic *7*, 129-145.

Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., *et al.* (2005). Impairment of starvation-induced and constitutive autophagy in Atg7-deficient mice. J Cell Biol *169*, 425-434.

Verheije, M.H., Raaben, M., Mari, M., Te Lintelo, E.G., Reggiori, F., van Kuppeveld, F.J., Rottier, P.J., and de Haan, C.A. (2008). Mouse hepatitis coronavirus RNA replication depends on GBF1-mediated ARF1 activation. PLoS pathog *4*, e1000088.

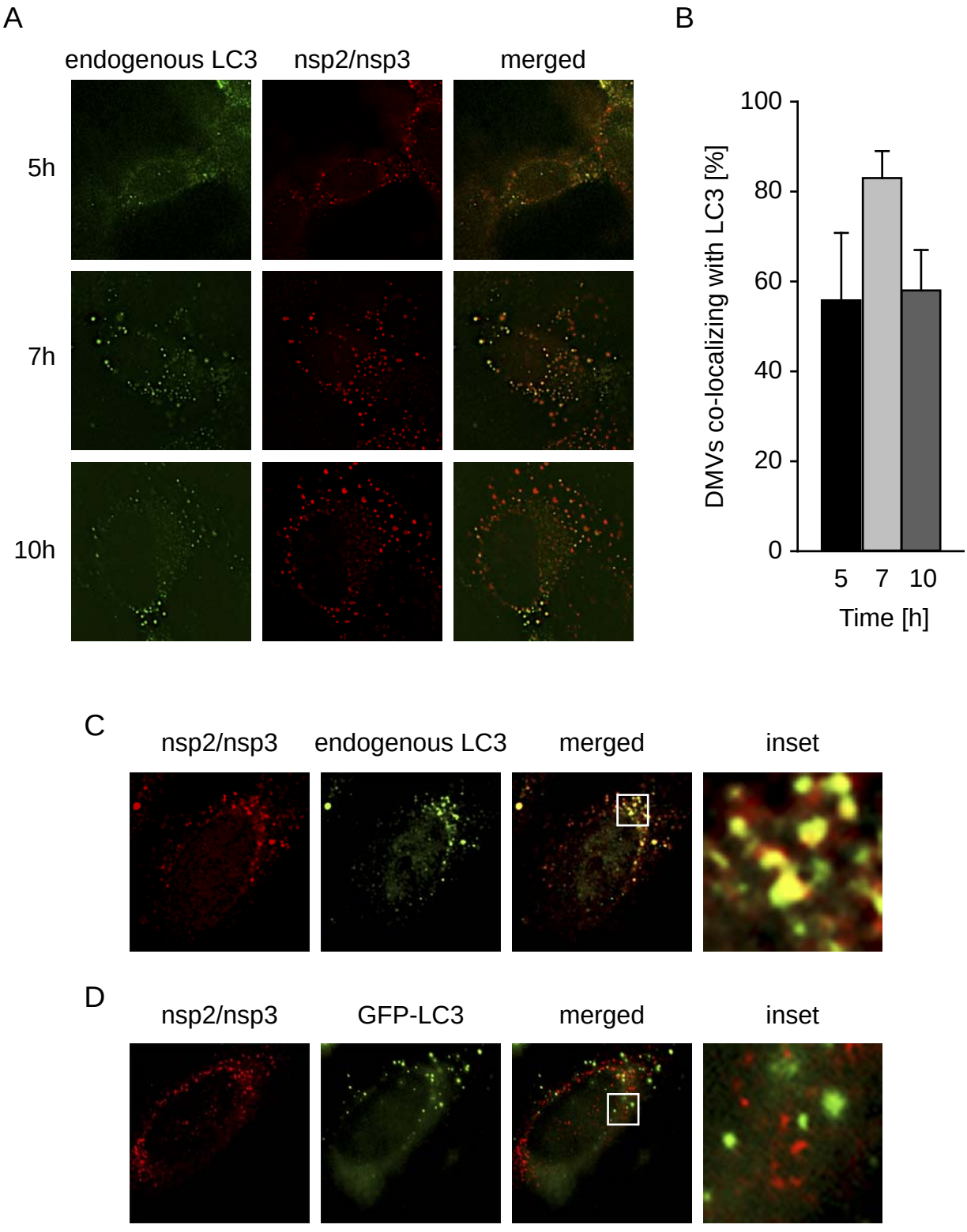

Figure S1, related to Figure 2. Endogenous LC3 co-localization with MHV-induced DMVs. A. HeLa-CEACAM1a cells were infected with MHV and processed for IF at 5h, 7h and 10h p.i. B. Summary statistics of the samples shown in panel A as the percentage of DMVs co-localizing with LC3. Error bars represent the standard error of the mean percentage from counting of 40 cells. C,D. HeLa cells were transfected with either a plasmid expressing GFP-LC3 (D) or mock-treated (C) and after 24h, infected with MHV-Srec. Cells were then fixed at 7h p.i and analysed by IF as in Figures 2A-2B. This analysis has been performed twice.

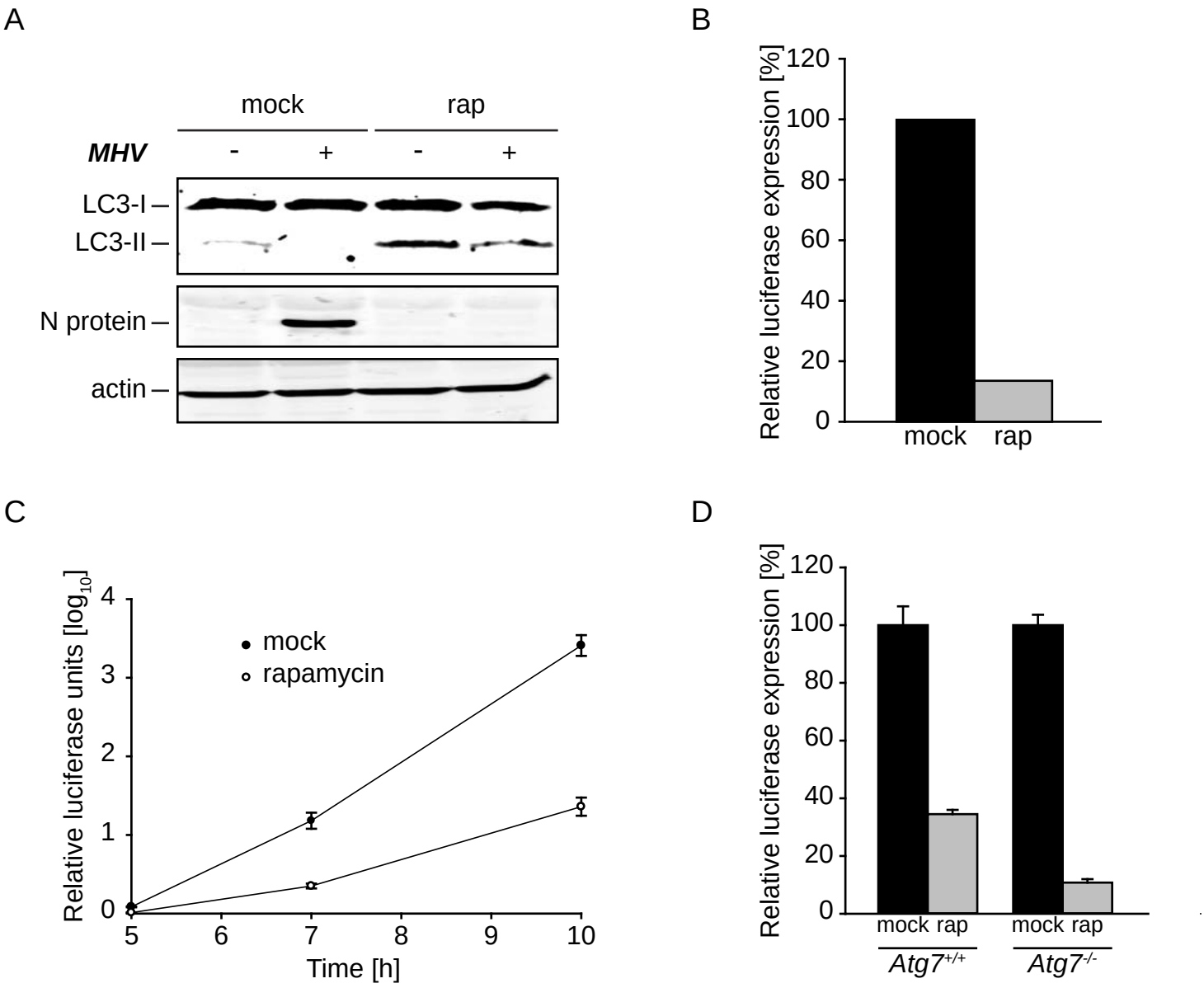

Figure S2, related to Figure 3. Rapamycin treatment affects MHV replication in an autophagy-independent way. A,B. HeLa-CEACAM1a cells grown in presence of protease inhibitors (10 mg/ml E64d and 28 mg/ml pepstatin A) for 1h were inoculated with MHV-2aFLS and 10 μM rapamycin was added 1h p.i. Subsequently, cell extracts were prepared at 7h p.i. and used to analyze (A) the N protein synthesis by Western blot and (B) the virus replication by measuring luciferase expression. This analysis was repeated twice. C. The experiment described in panel B was repeated in a time-course manner and luciferase expression determined at 5h, 7h and 10h p.i. D. The experiment described in panel B was also performed in *Atg7*$^{+/+}$ and *Atg7*$^{-/-}$ MEF, and repeated 3 times. It shows that rapamycin inhibits MHV replication independently of the presence of an intact autophagy machinery. Error bars in panles B, C and represent the standard deviations between experiments.

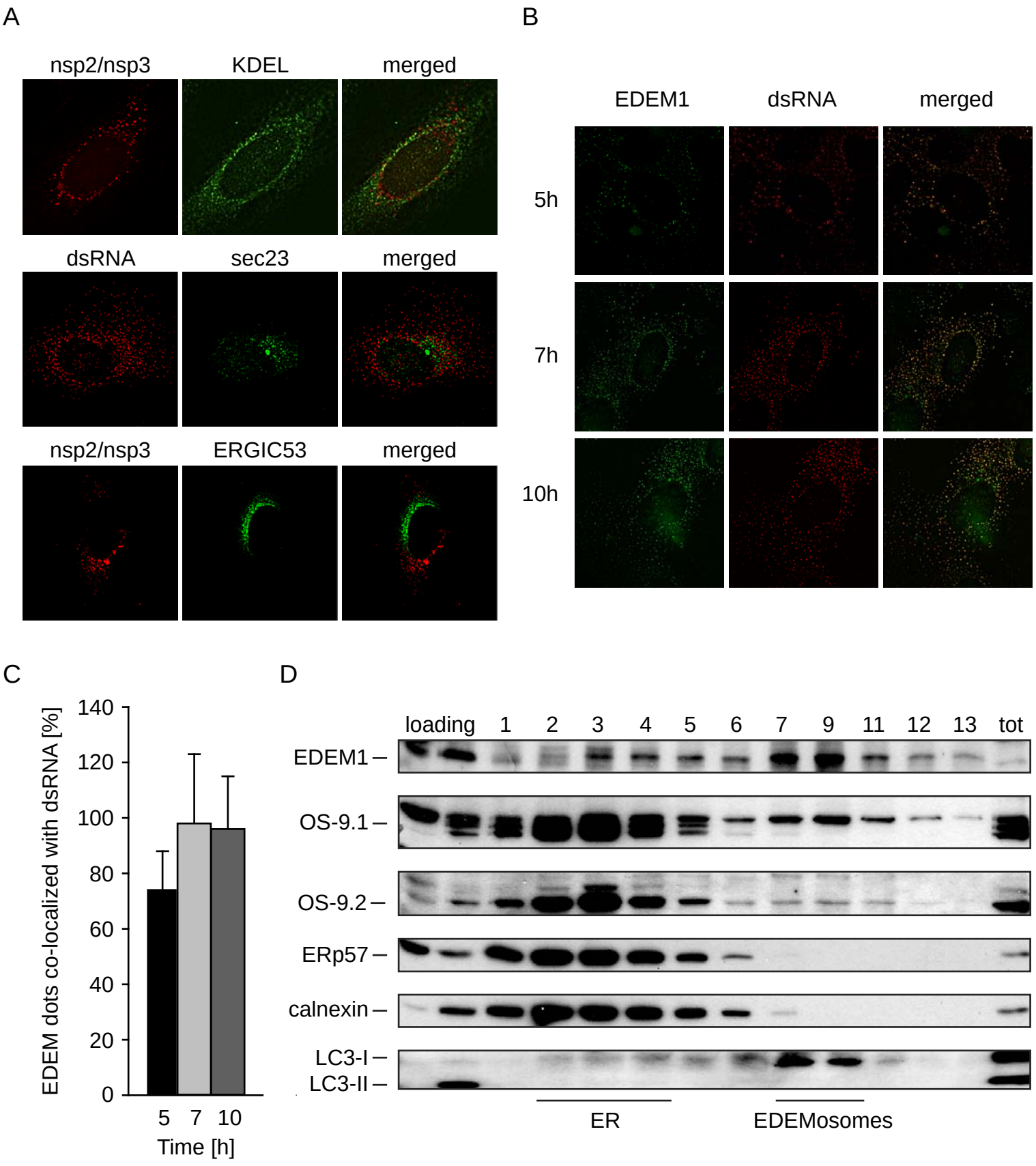

Figure S3, related to Figure 2. Relationship between CoV-induced DMVs and EDEMosomes. A. HeLa cells were infected with MHV-Srec and fixed at 7h p.i. before being processed for IF with antibodies against the dsRNA or nsp2/nsp3 and the KDEL tetrapeptide (ER), Sec23 (ER exit sites and COPII vesicles) or ERGIC-53 (ERGIC and *cis*-Golgi). These analyses have been carried out twice. B. HeLa-CEACAM1a cells were infected with MHV and processed for IF at 5h, 7h and 10h p.i. C. Summary statistics of the samples shown in panel B as the percentage of EDEM1 co-localizing with MHV-induced DMVs. Error bars represent the standard error of the mean percentage from counting 40 cells. D. A cell extract (tot) obtained from MEF was fractionated on a Optiprep gradient and fractions were analyzed by Western blot with antibodies against EDEM1, OS-9, LC3, calnexin and ERp57. This subcellular fractionation experiment has been repeated 5 times with identical results.

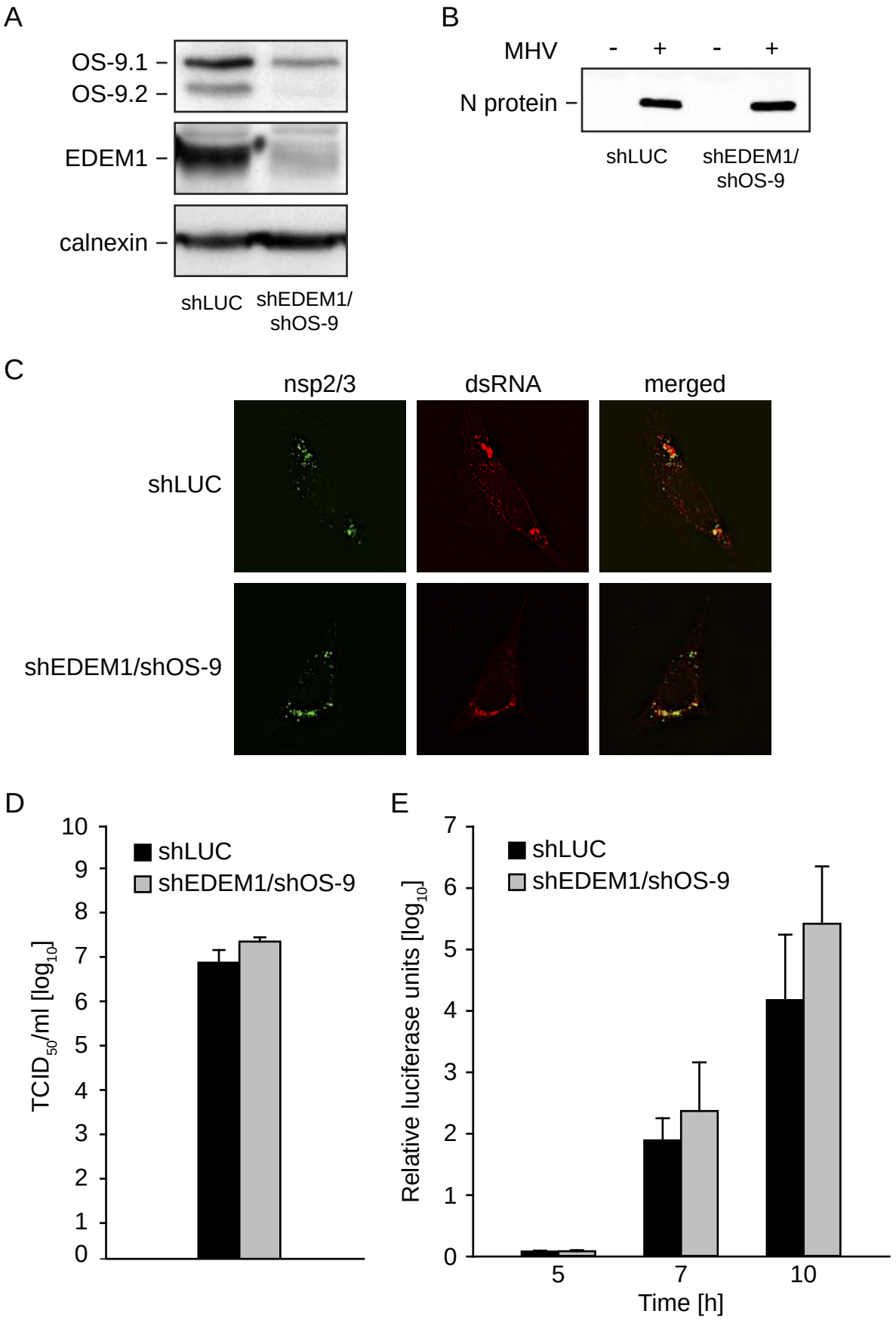

Figure S4, Related to figure 3. EDEM1 and OS-9 are not required for MHV infectivity. A. Expression levels of EDEM1 and OS-9 in shLUC and shEDEM1/shOS-9 HEK293 cells assessed by Western blot. Calnexin was used as the loading control. B,C,D. The shLUC and shEDEM1/shOS-9 HEK293 cell lines were infected with MHV-Srec for 7h. Subsequently, cell extracts were prepared to assess the synthesis of the N structural protein (B), IF was performed to monitor DMV biogenesis (C) and the titer of a virus stock (D) was determined as described in Figure 1D. This experiment was performed twice and error bars in D represent the standard deviation between experiments. E. The shLUC and shEDEM1/shOS-9 HEK293 cell lines were also infected with MHV-2aFLSrec and luciferase expression was measured at 5h, 7h and 10h. The experiment was done in triplicate and error bars represent the standard deviations between samples.