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Nipah virus matrix protein influences fusogenicity and is essential for particle infectivity and stability

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Running title: NiV M protein required for infectivity and stability

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23 **ABSTRACT**

24 Nipah virus (NiV) causes fatal encephalitic infections in humans. To characterize the role of
25 the matrix (M) protein in the viral life cycle, we generated a reverse genetics system based
26 on the NiV Malaysia strain. Using an eGFP-expressing matrix (M) protein-deleted NiV, we
27 observed a slightly increased cell-cell fusion, slower replication kinetics and significantly
28 reduced peak titers compared to the parental virus. While increased amounts of viral
29 proteins were found in the supernatant of cells infected with M-deleted NiV, the infectivity-
30 to-particle ratio was more than 100-fold reduced, and the particles were less thermostable
31 and of more irregular morphology. Taken together, our data demonstrate that the M protein
32 is not absolutely required for the production of cell-free NiV, but is necessary for proper
33 assembly and release of stable infectious NiV particles.

34

35 **IMPORTANCE**

36 Henipaviruses cause a severe disease with high mortality in human patients. Therefore,
37 these viruses can only be studied in BSL-4 laboratories, making it more challenging to
38 characterize their life cycle. Here we investigated the role of the Nipah virus matrix protein
39 in virus-mediated cell-cell fusion and in the formation and release of newly produced
40 particles. We found that even though low levels of infectious viruses are produced in the
41 absence of the matrix protein, it is required for the release of highly infectious and stable
42 particles. Fusogenicity of matrix-less viruses was slightly enhanced, further demonstrating
43 the critical role of this protein in different steps of Nipah virus spread.

44

45 **INTRODUCTION**

46 Nipah virus (NiV) is a zoonotic paramyxovirus in the *Henipavirus* genus that originates from
47 *Pteropus* bats. It causes sporadic outbreaks of deadly encephalitic disease in humans in
48 Malaysia, Singapore, India and Bangladesh (1, 2). Cross-reactive antibodies against NiV and
49 other related henipaviruses have been detected in bats and pigs as far afield as Africa and
50 other parts of Southeast Asia, indicating that these viruses circulate quite widely (3-10).
51 NiV entry and cell-to-cell spread is driven by two transmembrane glycoproteins, the
52 attachment (G) and the fusion (F) proteins, that are exposed on the surface of viral particles
53 and on infected cells to mediate attachment to the host cell receptor and membrane fusion,
54 respectively. The viral matrix (M) protein associates with the inner leaflet of the plasma
55 membrane mediating the contact between the ribonucleoprotein (RNP) complex and the
56 surface glycoproteins. Though the detailed role varies between different viruses,
57 paramyxoviral M proteins are generally considered the main drivers of assembly (11).
58 Supporting the idea of a critical role in virus particle formation and budding, NiV M protein
59 forms virus-like particles when expressed on its own (12, 13), and it drives apical assembly
60 and budding of NiV virions in polarized epithelial cells (14). Trafficking of the NiV M is a
61 complex process involving transit through the nucleus (15-18), despite replication occurring
62 exclusively in the cytoplasm. When NiV M protein nuclear localization or export signals are
63 interrupted, or if ESCRT pathway-interacting late domains are disrupted, NiV M proteins lose
64 their ability to accumulate at the plasma membrane and no longer generate virus-like
65 particles (12, 17, 19). Aside of the M protein, the NiV glycoproteins appear to also possess
66 intrinsic budding capabilities (13), but their roles in viral egress remain unresolved.

67 So far, only two paramyxoviruses, measles virus (MV) and human respiratory syncytial virus
68 (HRSV), have been successfully rescued without transcomplementation by plasmid-encoded

69 M protein (20, 21). We show here that a recombinant eGFP-expressing M protein-deficient
70 NiV (NiVeGΔM) could be recovered and propagated in the absence of any exogenous M
71 expression. NiVeGΔM was detected in the culture supernatant, though virus titers were up
72 to 1000-fold lower than for the parental wild-type virus, and cell-free viruses were less
73 stable at 37°C. NiVeGΔM also displayed enhanced fusion kinetics, suggesting that the M
74 protein plays a role in downregulation of the F/G-mediated cell-cell fusion. Taken together,
75 our data show that the M protein plays an important role for the correct assembly of
76 infectious cell-free NiV particles and influences the kinetics of cell-associated spread of NiV
77 infection.

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81 **MATERIALS AND METHODS**

82 **Cells and viruses.** Vero 76 cells (ATCC #CRL1587) and 293 cells (ATCC #CRL1573) were
83 cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS),
84 100 U of penicillin/ml, 0.1 mg of streptomycin/ml, and 4 mM glutamine (all Life
85 Technologies). All virus recovery and NiV infection experiments were performed in the BSL-4
86 containment at the Institute of Virology, Philipps University of Marburg, Germany.

87 **Generation of NiV full-length cDNA plasmids.** Expression plasmids containing the
88 nucleoprotein (N), phosphoprotein (P), and polymerase (L) protein were a kind gift of Dr.
89 Markus Czub. To amplify fragments spanning the leader, trailer, and untranslated regions
90 (UTR), RNA isolated from Vero cells infected with the NiV Malaysia strain (GenBank Acc. No.
91 NC 002728) was reverse transcribed using Superscript III (Invitrogen, Burlington, ON) using
92 random hexamer primers. For the internal UTRs, the primers were chosen to include a

93 unique or partially unique restriction site in each flanking gene. To introduce the enhanced
94 green fluorescent protein (eGFP) in an additional transcription unit between G and L, the PM
95 UTR was duplicated, and inserted between the G and eGFP open reading frames, yielding
96 pBRT7-NiVeG. The M gene-deleted derivative pBRT7-NiVeGΔM was produced by deleting
97 the M open reading frame except for the stop codon to assure the rule-of-six.

98 **Recovery of recombinant viruses.** To recover recombinant Nipah viruses,
99 semiconfluent 293 cells in 6-well plates were infected with MVA-T7 at a multiplicity of
100 infection (MOI) of 1. After 1h at 37°C, medium was changed to 500µl OptiMEM. Then, cells
101 were transfected with 0.75µg pTM1-NiV N, 0.05µg pTM1-NiV P, 0.4µg pTM1-NiV L, and 5µg
102 pBR/T7-NiVeG or pBR/T7-NiVeGΔM, respectively, using Lipofectamine 2000 (Life
103 Technologies). After 3 – 4 hours at 37°C, medium was changed to DMEM 2% FCS with
104 glutamine and antibiotics. If necessary, fresh 293 cells were added and medium was changed
105 after 1 and 3 days, and the supernatant was transferred onto Vero cells after 6-9 days. Virus
106 was harvested when 70 – 90% of the Vero cells showed cytopathic effects.

107 **Virus titration, particle stability, and growth kinetics.** Virus titers were quantified by
108 limited dilution method and expressed as 50% tissue culture infections doses ($TCID_{50}$). To
109 evaluate the particle stability, these titers were compared with titers obtained after 1 and 5
110 day incubation at 4°C or 37°C, respectively. For growth kinetics, confluent Vero cells seeded
111 in 6-well plates were infected at a MOI of 0.001. After 1 h at 37°C, cells were washed 3 – 5
112 times, and samples from the supernatant were collected (t0). Additional samples were
113 collected and titrated after 24, 48, and 72 h. To compare cell-free and cell-associated virus
114 titers, the cell culture supernatant was removed after 48 h, cleared for 10 min at 15,000g
115 and used to determine cell-free virus titers. Infected cells were scraped into OptiMEM and
116 frozen at -80°C. After rapid thawing at 37°C, cell lysates were cleared by low-speed

117 centrifugation, and cell-associated infectivity in the supernatants released by the freeze-
118 thaw cycle was quantified. Viral titers ($n \geq 3$) were compared using an unpaired t-test,
119 performed in Microsoft Excel using the "T.TEST" function.

120 **Live cell imaging.** To characterize the dissemination of the different viruses, Vero
121 cells seeded in 35-mm µ-dishes (Ibidi, Munich) were infected with NiVeG or NiVeGΔM at a
122 MOI of 0.005. After 1 h at 37°C, medium was replaced by CO_2-independent Leibovitz's
123 medium without phenol red (Life Technologies) with 100U/ml penicillin and 100µg/ml
124 streptomycin, 20% FBS, and 400µM 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic
125 acid (Trolox; Sigma). Live cell time-lapse experiments were started at 16 h after infection and
126 recorded with a Leica DMI6000B microscope using a 20x objective equipped with a remote
127 control device to operate the microscope from outside the BSL-4 facility. Pictures were taken
128 every 30 min and processed with Leica LAS AF software. The increase in size of a syncytium
129 as a parameter for fusion kinetics was determined by measuring the area of 12 individual
130 syncytia at different time points using ImageJ (http://rsbweb.nih.gov/ij). To calculate the
131 relative increase, the area of the syncytium at 17 h after infection was set as 1.

132 **MTT assay.** The MTT assay was performed according to the manufacturer´s protocol
133 (Thermo Fisher Vybrant MTT Cell Proliferation Assay Kit). Briefly, Vero cells grown in a 96-
134 well plate were infected with NiVeG or NiVeGΔM at a MOI of 0.001 or 0.01. After 2 days,
135 medium was replaced by PBS containing 1 mM MTT. After incubation for 4 h at 37°C,
136 supernatants were removed, mixed with DMSO and further incubated for 10 min at 37°C
137 before absorption was measured at 562 nm using a PHOmo Microplate reader. To calculate
138 the relative cytotoxicity, the absorbance of uninfected cells was set to 1.

139 **Quantification of NiV-N RNA.** Viral RNA was extracted from supernatants of NiVeG or
140 NiVeGΔM-infected Vero cells using the RNeasy kit (Qiagen) and reverse transcribed using
141 Revert Aid H Minus Reverse Transcriptase (Fermentas). Real time PCR reactions were
142 performed in triplicates with a StepONE Plus cycler using QuantiFast SYBR green PCR Master
143 Mix (Applied BioSystems) and NiV N specific diagnostic primers. Genome numbers (2^{-Ct}), and
144 the infectivity-to-particle ratio calculated by dividing the titer ($TCID_{50}$/ml) by 2^{-Ct}, was
145 normalized to the values obtained for NiVeG (set as 1).

146 **Virus purification and Western blot analysis.** To evaluate viral protein expression
147 levels, Vero cells were infected with NiVeG or NiVeGΔM at a MOI of 0.001. After 48 h, the
148 supernatant was harvested and precleared by centrifugation for 10 min at 15,000g. Virus
149 particles were then isolated by ultracentrifugation through a 20% Sucrose cushion for 1.5 h
150 at 150,000g and subsequent resuspension of the pellet in 30 µl 1% sodium dodecylsulfate
151 (SDS, Sigma) in phosphate-buffered saline (PBS, Life Technologies). Cell lysates were
152 collected at the same time by scraping the cells into 1% SDS in PBS. Two % of the cell lysate
153 and 33% of the virus pellet were then separated by reducing SDS-PAGE and transferred onto
154 nitrocellulose. Membranes were incubated with polyclonal rabbit antisera directed against
155 NiV G, F, and M peptides (G1126, F631, M1321; immunGlobe, Himmelstadt, Germany),
156 followed by a biotin-labeled anti-rabbit antiserum and horseradish peroxidase-conjugated
157 streptavidin (Amersham). Bands were detected using a ChemiDoc (BioRad). To stain NiV N,
158 membranes were incubated with a polyclonal anti-NiV guinea pig serum (22). Primary
159 antibodies were detected with an IRDye700-conjugated anti-guinea pig secondary antibody
160 (LI-COR) and visualized with an Odyssey Imager (LI-COR).

161 **Proteinase K protection assay.** 2 x 10^6 Vero cells were infected with NiVeG or
162 NiVeGΔM at a MOI of 0.001. After 48 h, supernatants were cleared for 10 min at 15,000g

163 and subsequently centrifuged for 1 h at 150,000g. Virus pellets were resuspended in 60µl
164 PBS. Twenty µl of the virus suspension was either left untreated (control), or was treated for
165 30 min at 37°C with Proteinase K at a final concentration of 0.1µg/µl in the absence or
166 presence of 1% Triton X-100. Digestion was stopped by the addition of 1 µg/µl PMSF.
167 Samples were then inactivated and subjected to Western blot analysis using the polyclonal
168 anti-NiV guinea pig serum.

169 **Electron microscopy.** For each virus, Vero cells grown to confluency in three 175 cm^2
170 flasks were infected with NiVeG or NiVeGΔM at a MOI of 0.005. After 48 h, virus particles
171 were purified as outlined above, and the pellets were resuspended in 150µl PBS. A drop of
172 purified virus suspension was added on Formvar-coated nickel grids and incubated for 5 min.
173 Then, samples were inactivated with 4% PFA for two days, and negative staining was
174 performed with 2% phosphotungstic acid. Alternatively, grids were immuno-stained using a
175 NiV-specific guinea pig serum and donkey anti-guinea pig secondary antibodies conjugated
176 with 12 nm colloidal gold (Jackson ImmunoResearch; USA). Samples were analyzed by using
177 a JEM 1400 transmission electron microscope at 120 kV.

178 **RESULTS**

179 **M gene-deleted NiV are released in the supernatant.** While non-infectious systems
180 have contributed importantly to the characterization of the henipavirus life cycle, several
181 fundamental questions can only be answered using recombinant viruses. So far, such
182 genetically modified recombinant NiV have been either used to analyze the functions of V,
183 W or C proteins (23-25), or to characterize chimeric viruses in which NiV genes were
184 exchanged by the homologous Hendra virus (HeV) genes (26). Here, we generated a
185 replicative M gene-deleted NiV to characterize assembly and budding of infectious cell-free
186 NiV particles in the total absence of M. Towards this, we generated a reverse genetics
187 system based on the strategy used for the related morbilliviruses (27, 28). The entire
188 genome of the NiV Malaysia strain was assembled in a low copy plasmid and flanked by a T7
189 promoter and a T7 terminator/hepatitis ∂ ribozyme cassette to ensure correct genome ends
190 (NiV, Fig. 1A). An additional transcription unit carrying the eGFP gene was introduced
191 between the G and L genes (NiVeG) to facilitate the detection of infected cells. To generate
192 an M-deleted virus (NiVeGΔM), the M open reading frame was deleted from the NiVeG
193 genomic plasmid (Fig. 1A).
194 All recombinant viruses could be recovered by transfecting the respective genomic plasmid
195 with T7 polymerase-driven expression plasmids for the N, P, and L proteins in 293 cells
196 previously infected with MVA-T7, which provided the T7 polymerase. Interestingly,
197 NiVeGΔM could be rescued and propagated without transcomplementation of plasmid-
198 encoded M protein. While replication efficacies of recombinant NiV and NiVeG were similar,
199 indicating that introduction of the GFP cassette did not affect virus growth (Fig. 1B),
200 NiVeGΔM was associated with ten- to thousand-fold lower titers (Fig. 1C). Since the maximal
201 cell-associated infectivity was similarly reduced (Fig. 1D), this cannot be explained by just a

202 budding defect in the absence of M. Instead, it indicates that the M protein is required for
203 proper assembly of infectious particles.

204 **Absence of M protein results in enhanced syncytia formation kinetics.** While
205 deletion of Sendai or measles virus (MV) M genes caused an increased fusogenicity and
206 resulted in the formation of much larger syncytia (20, 29), no influence on the cytopathic
207 effect was observed for a human respiratory syncytial virus (HRSV) lacking the M protein
208 (21). Phase contrast microscopy suggested a slightly enhanced fusogenicity of NiVeGΔM.
209 However, because of the heterogeneous sizes of syncytia at any steady state time point after
210 infection, the differences in average syncytia sizes were not statistically significant (data not
211 shown). We therefore performed a live-cell imaging analysis of Vero cells infected with
212 NiVeG and NiVeGΔM. By monitoring individual syncytia over two days, we analyzed fusion
213 kinetics to determine the influence of M deletion on the progression of NiV-mediated cell-
214 cell fusion. For both viruses, first syncytia encompassing 2-5 cells were detected 17 h after
215 infection (Fig. 2A). There was a gradual increase in syncytia sizes over time for both viruses,
216 but fusion kinetics was more pronounced for NiVeGΔM (Fig. 2B). Though syncytia generally
217 grew faster due to the increased fusogenicity of NiVeGΔM, this did not result in an enhanced
218 cytotoxic effect. Cell viability of NiVeG and NiVeGΔM-infected cells at 24 h and 48 h p.i. did
219 not differ significantly (Fig. 2C). As in Vero cells, we observed an enhanced fusogenicity of
220 NiVeGΔM in A549 cells (data not shown). Together, these results illustrate that the NiV M
221 protein modulates glycoprotein-mediated cell-to-cell fusion to a greater extent than the M
222 protein of HRSV, albeit to a lesser extent than the M proteins of Sendai virus or MV.

223 **M protein deletion causes formation of less infectious particles with reduced**
224 **stability.** To determine if reduced NiVeGΔM titers are the result of less efficient particle
225 formation, or of a difference in particle infectivity, we compared the amount of viral RNA in

226 the supernatant of NiVeG or NiVeGΔM-infected cells, in which infectious titers of the latter
227 were about 100-fold reduced. Real-Time RT-PCR analysis (qPCR) with NiV-N specific primers
228 revealed an increased relative particle/genome number in the supernatant of NiVeGΔM-
229 infected cells (Fig. 3A, left panel). Setting the infectivity-to-particle ratio of NiVeG to 1,
230 NiVeGΔM thus yielded a relative infectivity between 0.001 and 0.01 (Fig. 3A, right panel). To
231 evaluate if differences in viral protein expression account for this observation, we analyzed
232 the NiV protein expression in infected cell lysates and the particle composition. As expected,
233 the M protein was absent in NiVeGΔM-infected cells and the resulting viral particles (Fig. 3B,
234 lines 3 and 4). While the viral protein content in infected cells was otherwise not affected by
235 the absence of M (Fig. 3B, lanes 1 and 3), the total amount of G, F, and N proteins in the
236 purified viral particle fraction was considerably higher for NiVeGΔM (Fig. 3B, lanes 4 and 2).
237 As shown in the Coomassie-stained gel (Fig. 3C), NiVeGΔM virus preparations not only
238 contained higher amounts of viral proteins but also a substantially increased amount of total
239 (cellular) proteins (Fig. 3C). Proteinase K (PK) digestion in the absence and presence of TX-
240 100 confirmed that particles with incorporated N protein are present in NiVeG and NiVeGΔM
241 supernatants (Fig. 3D). However, compared to PK-treated NiVeG particles, the relative
242 amount of N protein in PK-digested NiVeGΔM particles was reduced. This might hint on a
243 compromised viral membrane integrity when M is absent.
244 To assess if M depletion not only affects the composition of cell-free virus preparations but
245 also their thermostability, we compared the infectivity of NiVeG and NiVeGΔM after
246 incubation at 4°C or 37°C. While infectious titers of both viruses remained almost stable for
247 5 days at 4°C, incubation for 24 h at 37°C had little effect on the parental NiVeG, but resulted
248 in a statistically highly significant tenfold drop in titers for NiVeGΔM (Fig. 3E). After 5 days at
249 37°C, infectivity of NiVeGΔM was reduced by more than hundredfold, while NiVeG titers had

250 only dropped tenfold (Fig. 3E). Taken together, this supports the idea that in the absence of
251 M, the coordinated assembly of largely cell protein-free virus virions is disturbed, resulting in
252 the production of cell-free NiVeGΔM particles with increased cellular protein incorporation
253 and lower particle stability.

254 **The M protein coordinates the budding process.** To gain more detailed insights in
255 the morphology of the released particles, we performed a negative-stain transmission EM
256 analysis of cell-free virus preparations from NiVeG and NiVeGΔM-infected cell supernatants
257 at 48 h after infection. Consistent with the detection of increased viral and cellular protein
258 amounts by Western blot analysis and Coomassie staining (Fig. 3B and D), virus preparations
259 pelleted from supernatants of NiVeGΔM-infected cells contained a dramatically increased
260 amount of vesicular material compared to NiVeG virus preparations (Fig. 4A and B). For a
261 more in depth characterization of the particle morphology, we analyzed NiVeG and
262 NiVeGΔM samples by immuno-EM using a polyclonal anti-NiV guinea pig antiserum. In both
263 virus preparations, we found particles heavily decorated with immunogold beads. Among
264 them were spherical, filamentous and pleomorphic virions, as previously described by Hyatt
265 et al. (30). Spherical NiVeG and NiVeGΔM particle sizes both ranged from 90 to 380 nm (Fig.
266 4C and D), and filamentous NiVeG and NiVeGΔM particles also had similar total sizes (Fig. 4E
267 and F). However, these elongated NiVeGΔM particles had a more irregular shape and
268 displayed some defects in the particle envelope (Fig. 4F, arrows), what supports the idea of a
269 compromised membrane integrity suggested by the PK digestion (Fig. 3D). Similar to
270 filamentous particles, NiVeGΔM particles with pleomorphic shapes differed from
271 pleomorphic NiVeG by a more irregular morphology, an uneven surface and some envelope
272 blebs (Fig. 4G and H), indicating a less coordinated budding process in the absence of M.

273

274 **DISCUSSION**

275 The characterization of NiV assembly in the total absence of the M protein has thus
276 far relied on the co-expression of recombinant proteins outside the authentic viral context.
277 To address this limitation, we established a reverse genetics system based on the NiV
278 Malaysia strain. Using the system, we were able to recover NiVeGΔM, an eGFP-expressing
279 derivative lacking the M protein transcription unit. While the fusogenicity of NiVeGΔM was
280 slightly enhanced, replication kinetics was significantly impaired. Together with the release
281 of high amounts of viral and cellular proteins and the severely reduced infectivity and
282 stability, our data indicate that the M protein is essential for proper NiV particle assembly,
283 consistent with its role in other paramyxoviruses (21, 31-33).

284 **Requirements for virus-like particle and infectious particle formation are different.**
285 Virus-like particles (VLPs) are frequently used to investigate assembly mechanisms of
286 enveloped viruses (34). For NiV, expression of the F or G proteins alone is sufficient to yield
287 VLPs in the supernatant, and the co-expression of M greatly increases the efficiency of VLP
288 formation (13, 35). This intrinsic budding activity of the F and G proteins is likely the origin of
289 the large amount of viral protein detected in purified supernatant of NiVeGΔM-infected
290 cells. However, the low infectivity-to-particle ratio indicates that these M protein-
291 independent budding activities only accidentally generate infectious RNP-containing
292 particles while mostly yielding non-infectious virus-like particles also found in F/G expression
293 systems. Furthermore, it is likely that the envelope of the few RNP-containing particles
294 produced in the absence of the M protein has a suboptimal composition of surface
295 glycoproteins, which may impair the virus entry and uncoating processes (11, 21). Whether
296 the role of the M protein in the assembly process is mainly to concentrate viral components

297 at specific sites at the plasma membrane or to ensure a defined stoichiometry of RNPs, G
298 and F proteins requires further investigation.

299 **Conserved and divergent functions of paramyxovirus M proteins.**

300 Despite their conserved role in assembly and budding, there are genus- and strain-specific
301 differences between paramyxoviral matrix proteins that do not allow exchanging M proteins
302 even between closely related viruses without affecting virus growth (36-38). In line with
303 these studies, a recent cross-complementation study of Yun et al. (26) demonstrated that
304 introduction of the Hendra virus M gene into NiV increased the replicative titers, likely as a
305 result of an increased budding activity of HeV M.

306 While some attempts to generate members of the *Paramyxoviridae* family without
307 complementation of functional M proteins were not successful (31-33), M protein-deleted
308 measles (MVΔM), and respiratory syncytial (M-null HRSV) viruses have been recovered (20,
309 21). While M-null HRSV was completely defective in virus budding (21), limited amounts of
310 infectious MVΔM were found in the supernatant (20), indicating a varying importance of the
311 M protein contribution among different genera.

312 In line with what has been reported for MVΔM (20), we observed an increased fusogenicity
313 for NiVeGΔM. Although the effect of M depletion on NiV-mediated cell-cell fusion is clearly
314 less pronounced, it may be speculated that as in MV infection (39, 40), NiV M downregulates
315 cell-cell fusion by interacting with the cytoplasmic portions of the NiV surface glycoproteins.
316 In contrast to the increased fusogenicity seen in M-deficient MV, Sendai virus and NiV,
317 deletion of the HRSV M protein did not result in enhanced fusion (21), suggesting that
318 paramyxovirus M proteins differentially influence cell-to-cell fusion. In polarized cell types,
319 the role of M proteins might be even more diverse. Due to the polarized nature of epithelial
320 and endothelial target tissues, viral proteins and RNPs are often specifically transported to

apical or basolateral membrane domains. In the case of NiV, NiV F and G proteins contain cytoplasmic sorting signals that lead to a basolateral targeting upon single expression. In the viral context, however, both glycoproteins are expressed in a more apical fashion. This redistribution is assumed to be caused by the NiV M that is selectively targeted to the apical surface of polarized cells (14, 41, 42). This M-driven apical accumulation of all viral components is thought to ensure efficient apical NiV budding while downregulating F/G-dependent lateral cell-to-cell fusion kinetics within the polarized cell monolayer. Future studies will determine the effect of M deficiency on unipolar NiV budding and fusion downregulation in polarized endothelial and epithelial cell types.

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465

466 **FIGURE LEGENDS**

467 **Fig. 1. Characterization of recombinant eGFP-expressing wildtype and M protein-deleted**
468 **NiV. (A)** Schematic drawings of NiV, NiVeG, NiVeGΔM full-length genomes in the cDNA
469 plasmids. For all plasmids, the T7 RNA polymerase promoter is located immediately
470 upstream of the genome, while the hepatitis δ ribozyme and T7 terminator are located
471 immediately downstream of the genome to ensure correct genomic ends.
472 **(B)** Comparison of wildtype NiV and NiVeG titers. Vero cells were infected with recombinant
473 wildtype NiV and NiVeG at a multiplicity of infection (MOI) of 0.001. Cell-free virus was
474 quantified by the limited dilution method at 24 and 48 h p.i., and titers are expressed as 50%
475 tissue culture infectious doses ($TCID_{50}$/ml) (n = 4). Error bars indicate the standard deviation.
476 **(C)** Comparative growth kinetics of NiVeG and NiVeGΔM. Vero cells were infected at a MOI
477 of 0.001. Cell-free virus titers were determined at 0, 24, 48 and 72 h (n = 3). Dotted line
478 indicates the detection limit (50 $TCID_{50}$/ml). An unpaired t-test was used to examine the
479 significance of differences from NiVeG, (**) $p < 0.01$.
480 **(D)** Comparison of cell-free and cell-associated virus titers. Vero cells were infected with
481 wildtype (wt) and M-deleted NiV (ΔM) at a MOI of 0.001. At 48 h p.i., cell supernatants were
482 harvested, cleared and titrated (cell-free). Cells were scraped into OptiMEM, subjected to
483 one freeze-thaw cycle, and virus titers were quantified (cell-associated).
484

485 **Fig. 2. Live-cell microscopy to monitor NiVeG and NiVeGΔM cell-to-cell fusion kinetics.**
486 Vero cells were infected with NiVeG and NiVeGΔM at a MOI of 0.005. Time-lapse microscopy
487 was started 16 h later, and GFP fluorescence signals were recorded every 30 min with a Leica
488 DMI6000B microscope. **(A)** Selected images of the same microscopic field are shown for the
489 indicated time points. Magnification 200x. Bar, 100 µm. **(B)** Quantitative fusion kinetics.

490 Areas of single syncytia were measured at different time points after infection (n=12). To
491 calculate the relative increase in size, the area of the syncytium after 17 h was set to 1.
492 Significance of difference between NiVeG and NiVeGΔM, $p < 0.1$.
493 **(C)** Effect of NiVeG and NiVeGΔM infection on cell viability. MTT assays were performed with
494 Vero cells infected at a MOI of 0.001 or 0.01 at 24 h and 48 h after infection. Data were
495 normalized to uninfected cells. Mean and SD are shown. Student´s t-test analysis did not
496 reveal any statistically significant differences.
497
498 **Fig. 3. Comparison of particle infectivity, protein composition, protease resistance, and**
499 **thermostability. (A)** Relative particle numbers and infectivity-to-particle ratios of NiVeG and
500 NiVeGΔM. Cell-free virus titers were quantified, and RNA was isolated from 100 µl
501 supernatant of infected cells after 48 h. Genomic RNA was quantified by qRT-PCR using NiV-
502 N specific primers. The relative particle/genome numbers (2^{-Ct}) normalized to NiVeG (set as
503 1) are shown in the left panel. The infectivity-to-particle ratio was calculated by dividing the
504 titer ($TCID_{50}$/ml) by genome numbers (2^{-Ct}) and was normalized to the values obtained for
505 NiVeG (set as 1) to get the relative particle infectivity (right panel). Error bars indicate the
506 standard deviation (n = 3). Statistical significance; (***) p <0,001.
507 **(B, C)** Viral protein content in infected cells and purified particles. Infected Vero cells were
508 lysed at 48 h after infection, and cell-free virus was purified from cell supernatants. For each
509 virus, 2 % of the total cell lysates and 33 % of the purified particles were separated on 10%
510 SDS-PAGE gels. **(B)** To specifically detect viral proteins, one gel was blotted to nitrocellulose.
511 Viral proteins were detected by Western blot analysis using NiV-M, -F, and –G-specific
512 antisera and HRP-conjugated secondary antibodies. Chemiluminescent signals were
513 recorded with a Chemidoc system (upper panel). NiV-N protein was visualized with a

514 polyclonal NiV-specific guinea pig serum and IRDye700-labelled secondary antibodies.
515 Signals were recorded with an Odyssey Imaging system (bottom panel). **(C)** To visualize all
516 proteins in cell lysates and virus pellets, one gel was stained with Coomassie blue.
517 **(D)** Proteinase K protection assay. At 48 h after infection, virus particles were pelleted from
518 clarified cell supernatants, suspended in PBS and either left untreated (w/o PK), or were
519 treated with proteinase K in the absence (+PK) or presence of Triton X-100 (+PK/TX100). The
520 samples were then analyzed by Western blot using polyclonal NiV-specific antibodies as
521 described above.
522 **(E)** Stability of NiVeG and NiVeGΔM infectivity at different temperatures. Cell-free virus was
523 incubated for the indicated times at 4°C or 37°C, respectively. Titers at day 0 were set as
524 100%, and used for the calculation of the relative loss in virus titers. Error bars indicate the
525 standard deviation (n = 3). Statistical significance; (*) $p < 0.05$, (***) $p < 0.001$.
526
527 **Fig. 4. Ultrastructural analysis of viruses from cell supernatants.** Vero cells were infected
528 with NiVeG and NiVeGΔM at a MOI of 0.005 for 48 h. Virus particles were purified from the
529 supernatant by centrifugation over a 20% sucrose cushion, fixed and inactivated for 48 h in
530 4% paraformaldehyde. **(A, B)** Samples were subjected to negative staining with 2%
531 phosphotungstic acid. Bars, 1 µm. **(C-H)** Virus preparations were analyzed by
532 immunoelectron microscopy using a NiV-specific polyclonal guinea pig antiserum and
533 secondary antibodies coupled with 12 nm colloidal gold beads, followed by negative
534 staining. **(C, D)** Spherical viral particles. Dashed lines indicate the positions of the measured
535 diameters. **(E, F)** Filamentous viral particles. Arrows show defects in the integrity of virus
536 envelope. **(G, H)** Pleomorphic particles. Arrowhead indicate membrane blebs. Bars, 200 nm.