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## **Formation of virus-like particles from human cell lines exclusively expressing influenza neuraminidase.**

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1 *Full title:* Formation of virus-like particles from human cell lines exclusively expressing  
2 Influenza neuraminidase.

3

4 *Running title:* Influenza NA alone can form virus like particles.

5

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8

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16

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21

22 **Summary:**

23           The minimal viral requirements for the generation of influenza virus like particle (VLP)  
24 assembly and budding was reassessed. Using neuraminidase from the H5N1 and H1N1  
25 subtypes, it was found that the expression of neuraminidase (NA) alone was sufficient to  
26 generate and release VLPs. Biochemical and functional characterization of the NA containing  
27 VLPs demonstrated that they were morphologically similar to influenza virions. The NA  
28 oligomerization was comparable to that of the live virus, and the enzymatic activity, while not  
29 required for the release of NA-VLP, was preserved. Together, these findings indicate that NA  
30 plays a key role in virus budding and morphogenesis and demonstrates that NA-VLPs  
31 represent a useful tool in influenza research.

32

33

34 **Introduction**

35           Influenza A viruses are lipid enveloped members of the *Orthomyxoviridae* family.  
36 They contain 8 negative-sense, single-stranded RNA segments encoding 10 viral proteins.  
37 Influenza virions are pleomorphic although generally their shape is roughly spherical with a  
38 diameter around <150 nm. However, larger [100-400 nm] influenza virions are also  
39 generated, as are filamentous forms (Fujiyoshi *et al.*, 1994).

40

41           Influenza viruses derive their lipid envelope by budding from the plasma membrane of  
42 the infected cells and progeny virions are normally not found inside the host cell. Therefore  
43 assembly and budding are the final, but essential steps in the virus life cycle. M1 matrix  
44 protein is the most abundant protein in the influenza virion and plays a critical role in both  
45 virus assembly and budding (reviewed in Nayak *et al.*, 2009). M1 affects virus assembly by  
46 interacting with the core viral ribonucleocapsid (vRNP) and cytoplasmic tail of  
47 transmembrane proteins, forming a bridge between the two layers, as well as recruiting the  
48 internal viral proteins and vRNA to the plasma membrane in a cooperative manner (Noda *et al.*  
49 *et al.*, 2006). In addition, M1 interacts with the lipid bilayer producing an outward bending of the

50 membrane and this has been postulated to be the major driving force of influenza budding  
51 since cells expressing M1 protein alone assemble into virus-like particles (VLPs) (Gómez-  
52 Puertas *et al.*, 2000; Latham & Galaeza, 2001).

53

54 Haemagglutinin (HA) and neuraminidase (NA) are the two major surface  
55 glycoproteins in the influenza viral membrane. HA binds to sialic acid receptors on the cell  
56 surface and mediates the fusion process (Matrosovich *et al.*, 2006); whereas NA cleaves the  
57 terminal sialic acids from the cell surface glycans to facilitate release of the progeny virus  
58 from the host cell and prevent aggregation of virus particles (Bucher & Palese, 1975; Air &  
59 Laver, 1989). NA is also essential in the initial stage of virus infection by enhancing HA-  
60 mediated fusion (Su *et al.*, 2009), helping the virus to penetrate the mucin barrier protecting  
61 the airway epithelium (Matrosovich *et al.*, 2004) and promoting virus entry (Ohuchi *et al.*,  
62 2006). Studies of mutant influenza viruses have previously shown that the cytoplasmic tails  
63 of HA and NA contribute to control virus assembly (Zhang *et al.*, 2000) and virus morphology  
64 (Jin *et al.*, 1997). The specific role of HA and NA in virus budding is controversial. COS-1  
65 cells expressing HA alone did not give rise to virus like particles (VLP) production (Gómez-  
66 Puertas *et al.*, 2000), however assembly and budding of a NA-deficient virus mutant could be  
67 rescued with exogenous bacterial NA to the level of wild-type virus (Liu *et al.*, 1995). These  
68 studies suggested that the presence of both viral proteins was not essential for virus budding.  
69 Nevertheless, studies of cells expressing recombinant viral proteins from an H3N2 virus  
70 demonstrated that both HA and NA were involved in the budding process (Chen *et al.*, 2007).

71

72 In this study, using a plasmid-driven VLP production system in human embryonic  
73 kidney (HEK-293T) cells, we looked at the minimal viral requirements for influenza virus  
74 assembly and budding. We showed that both H5 HA and N1 NA can be the driving forces for  
75 virus budding, whereas M1 only had a limited contribution. In addition, we demonstrated that  
76 expression of NA alone could lead to the budding of particles. The influenza VLPs with NA  
77 (NA-VLPs) were morphologically similar to influenza virions and had an intact sialidase

88 enzymatic activity. Comparable results in generating NA VLPs were found with NA from both  
89 seasonal and pandemic H1N1 and avian H5N1 viruses.

80

## 81 **Results**

82 **Determination of minimal viral requirement for VLP formation and release.** In  
83 order to determine which viral structural protein(s) is/are essential for VLP-formation and  
84 release, HEK-293T cells were transfected with plasmids encoding for H5N1 proteins HA, NA  
85 and M1 singly, or in combination. Protein composition of the VLPs released into the culture  
86 medium was analyzed by western blotting (Fig.1a) and budding of VLPs from the transfected  
87 cells was visualized by transmission electron microscopy (Fig. 2). None of the transfections  
88 affected the protein expression system as indicated by analysis of the glyceraldehyde 3-  
89 phosphate dehydrogenase (GAPDH) housekeeping protein expression. Co-transfection of  
90 genes coding for different viral proteins had a minimal impact on individual expression level  
91 as shown in cell lysates (Fig. 1a). The expression of the HA, NA and M1 in the cytosol  
92 appeared to be comparable. Release was quantified by densitometric analysis of the  
93 Western blots and expressed as a percentage of overall production (cumulative both from  
94 lysates and in supernatant). VLPs were not detected by western blot from the culture  
95 medium of cells expressing HA alone unless exogenous bacterial NA was added (Fig. 1a,  
96 lanes 1 vs. 2), suggesting that VLPs budding from HA-expressing cells could not be released  
97 without exogenous NA as confirmed by VLP aggregation on the cell surface in TEM (Fig. 2a  
98 vs. 2c). When M1 was expressed alone, a small amount of the protein was detected in the  
99 supernatant and no significant change was observed by addition of exogenous NA (Fig. 1a,  
100 lanes 4 & 5). However, we were not able to find VLP structures from cells expressing M1  
101 alone (Fig. 2e) When HA and M1 were co-expressed, VLPs were released into the  
102 supernatant only in the presence of exogenous bacterial NA (Fig. 1a, lanes 7 & 8) with an  
103 increase of HA and M1 content when compared to their single-expression. Interestingly,  
104 release of both HA and M1 was significantly enhanced by the co-expression of NA (Fig. 1a,  
105 lanes 3, 6 & 9). Also, VLPs containing NA were easily detected from the cells expressing NA

106 exclusively (Fig. 1a, lane 10) and could be widely observed by TEM (Fig. 2d & 2f). These  
107 results confirmed the requirement of the NA enzymatic activity for the HA-containing VLP  
108 release, but also suggested a role of NA in VLP budding. Comparison of protein expression  
109 in the cell lysates as well as in the supernatant of infected or HA/NA/M1 transfected cells  
110 showed that HA and NA had similar expression levels (Fig. 1b), however the amount of M1  
111 released with the budding VLPs in the supernatant was approximately half of that found in  
112 virus infected cells. This was compatible with previous reports that M1 requires interaction  
113 with other viral proteins, such as the structural proteins HA/NA (Wang et al., 2010), as well  
114 as with viral RNPs which are absent from VLPs. Using TEM, the VLPs were pleomorphic  
115 with spherical or filamentous forms (Fig. 2b & 2f), similar to influenza virions.

116

117 **Physical and functional characterization of NA-VLPs produced in cells**  
118 **expressing influenza virus NA alone.** NA from the H5N1 subtype and from seasonal H1N1  
119 and pandemic H1N1 subtypes were included in this study. Culture medium of cells  
120 expressing the NA protein alone was harvested for detection of released particles. FLAG-  
121 tagged NAs were used to allow the detection with anti-FLAG antibodies of the protein from  
122 different viral origins at the same antibody binding affinity. No difference of VLP formation  
123 efficiency was observed between tagged and non-tagged NA (data not shown). Furthermore,  
124 similar particles were seen by TEM for all three subtypes we studied (Supplementary data  
125 S1). Sucrose gradient (20-60 %) centrifugation analysis demonstrated the presence of NA-  
126 VLPs in the intermediate pellet fractions between 30-50 % sucrose (Fig. 3 b-d), which was  
127 similar to the result observed with influenza A/WSN/33(H1N1) virions (Fig. 3a). In contrast,  
128 purified NA proteins were only present in lighter fractions (~20 % sucrose) of the gradient  
129 (Fig. 3e). In addition, NA proteins either purified or on the VLP surface or from the virus were  
130 functionally active as shown using a neuraminidase activity assay (Fig. 3f). The apparent  
131 shift of one fraction towards a higher concentration of sucrose for the virus versus the VLP  
132 was probably due to the presence of nucleic acid in the virus (RNPs) decreasing slightly their  
133 buoyancy.

134

135 Kinetic analysis of the NA-VLP production showed that it was continuous over at least  
136 60 hours post-transfection with continuous accumulation of the NA-VLP in the supernatant  
137 (Fig. 4a) when using NA from both pandemic H1N1 or from H5N1. Cleavage of caspase-3  
138 was not detected in all time-points indicating there was no evidence of apoptosis (Fig. 4b).  
139 Electron micrographs of transfected cells also failed to manifest features of apoptosis during  
140 the course of the experiment.

141

142 We found that sole expression of NA (from H5N1) was sufficient to promote release of  
143 NA-VLP (Fig. 5). A mutation of NA in position 262 (E to D) which abrogated the sialidase  
144 activity as well as treatment with Oseltamivir (sialidase inhibitor) failed to affect VLP  
145 formation. As expected, co-expression of HA (H5 from H5N1) increased the release of NA  
146 probably by the formation of heterochimeric VLP (HA/NA-VLP), but release of HA-containing  
147 VLP was then dependent on sialidase activity.

148

149 Immunoblotting analysis of VLPs showed that the NAs on the particle surface were in  
150 multimeric form, including the monomer (~55 kDa), dimer (~110 kDa), trimer (~165 kDa) and  
151 tetramer (~220 kDa) (Fig. 6). Multimeric NA complexes on the VLPs were denatured into  
152 monomer by heating in SDS loading buffer with chaotropic agent (urea) or reducing agent  
153 (DTT) (lanes 1 to 3). Upon pre-treatment of DTSSP before the denaturing step, multimeric  
154 NA complexes were protected from SDS and urea, and partially from DTT (lanes 4 to 6).  
155 Similar multimeric NA complexes were detected in the H1N1 virions but the detection failed  
156 in the samples pre-treated with DTSSP (lanes 7 & 8), probably due to the inability of the  
157 antibody to recognize cross-linked antigens. Samples from highly pathogenic H5N1 virus  
158 could not be analyzed as protocols for inactivating the infectivity of the highly pathogenic  
159 virus H5N1 so that these preparations could be taken out of bio-safety level-3 containment  
160 for further analysis compromises oligomer integrity.

161

162 Desialylation of cell surface sialic acids in cells expressing NA was then studied by  
163 lectin binding (Supplementary data S2). A weak SNA binding was observed in HEK-293T  
164 cells, indicating low level of  $\alpha$ -2,6 linked sialic acid on the 293T cells, and this disappeared in  
165 cells expressing any of the NAs studied. Similar loss of cell-surface sialic acids was seen in  
166 NA VLP expressing A549 or MDCK cells which express more  $\alpha$ -2,6 sialic acid (data not  
167 shown). MAA binding confirmed the presence of  $\alpha$ -2,3 linked sialic acid on mock transfected  
168 293T cells and this was significantly decreased in NA-expressing cells. Cleavage of sialic  
169 acids by sialidase is expected to expose the underlying Gal-GalNAc and this was  
170 demonstrated by the increased binding of PNA to NA-expressing cells.

171

## 172 **Discussion**

173 Influenza assembly, budding and release are the last but important steps in the  
174 replication cycle. VLP production assays using different systems have been developed to  
175 determine the viral proteins involved in the virus budding (Gómez-Puertas *et al.*, 2000;  
176 Latham & Galaeza, 2001; Neumann *et al.*, 2000). Here we investigated the minimal viral  
177 components required for assembly and budding of H5N1 influenza virus using a plasmid-  
178 driven VLPs formation system similar to that described in a previous study on H3N2 virus  
179 (Chen *et al.*, 2007).

180

181 Previous findings about the major viral proteins responsible for the virus budding have  
182 been contradictory. It was suggested that M1 plays the major role in driving virus budding  
183 from the cellular membrane (Gómez-Puertas *et al.*, 2000), whereas Chen and his colleagues  
184 found that M1 is not essential for the process but that HA and NA were necessary (Chen *et al.*,  
185 2007). A recent publication (Wang *et al.*, 2010) confirmed that M1 by itself fails to form  
186 virus-like particles, probably due to a lack of membrane-targeting signal and therefore  
187 requires the interaction with other viral proteins to be incorporated in the budding virions. In  
188 our study, although small amounts of M1 were released from the cells expressing M1 alone,  
189 no budding particle was observed by electron microscopy (Fig. 2e). A similar finding was



190 recently reported in which it was suggested that M1 was only nonspecifically secreted into  
191 the supernatant rather than released in the form of VLP (Tscherne et al., 2010). Nevertheless,  
192 co-expression of M1 increased the level of both HA and NA incorporated into the VLPs  
193 (compare Fig. 1a, lanes 2 & 8; lanes 6 & 10), suggesting that M1 helps the virus budding,  
194 probably by pushing the inner side of the cellular membrane.

195

196 VLP formation in cells transfected with HA alone or HA/M1 was completely dependent  
197 on the addition of exogenous NA. In the absence of exogenous NA, HA-containing VLPs  
198 were aggregated on the cell surface and not released into the medium (Fig. 1 & 2).  
199 Exogenous NA cleaved the cell surface sialic acids allowing the VLPs to be released,  
200 therefore both HA and M1 became detectable in the medium. These results suggested that  
201 the expression of HA could provide a driving force to trigger the production of VLP, although  
202 exogenous NA was necessary for the release of the particles.

203

204 Previously it has been suggested that NA was important for virus morphogenesis.  
205 Studies using influenza virus lacking cytoplasmic tails of HA or NA (HA<sup>-</sup>/NA<sup>-</sup>) demonstrated  
206 that NA<sup>-</sup> virus particles had an elongated morphology but not HA<sup>-</sup> virus (Jin *et al.*, 1997).  
207 Introduction of mutations in both the transmembrane and cytoplasmic domains of NA  
208 confirmed that NA is critical to control the virus shape, size and titer (Barman *et al.*,  
209 2004). Studies of mutant virus containing a large internal deletion in NA gene have shown  
210 they can assemble and bud similarly to wild-type virus (Liu *et al.*, 1995). However, our data  
211 has shown for the first time that expression of NA alone in HEK-293T cells could provide a  
212 driving force for the formation of extracellular NA-containing particles and this finding was  
213 seen with NA from different influenza viruses of the N1 subtype and demonstrated in at least  
214 two additional human cell-lines (A549 and HeLa, data not shown). It is also important to note  
215 that the release of M1 and HA was greatly enhanced by the co-expression with NA to a level  
216 which was not acquired by high concentration of exogenous NA, indicating that NA is likely to  
217 be a major force in driving virus budding. However, whereas Chen et al. (2007) reported that

218 only small amounts of NA were released when this glycoprotein was expressed on its own,  
219 our data showed that a large amount of NA was released. This discrepancy is probably due  
220 to the difference in influenza virus subtype used in the two studies (N2 in Chen et al., versus  
221 N1 here). In fact we did observe that N2 subtype NA was expressed to much lower level than  
222 N1 and that N1 from human seasonal H1N1 was expressed less than the highly pathogenic  
223 avian H5N1 (Supplementary data S3).

224

225 Furthermore, using a potent sialidase inhibitor and a point mutation (E262D) in NA  
226 that inactivates the catalytic site of the sialidase (Huang et al., 2008), we could show that  
227 neuraminidase activity of the NA was not necessary for the release of NA-VLP in cells (Fig.  
228 5).

229

230 To characterize the NA-VLPs, biochemical, physical, morphological and functional  
231 studies were done, including sucrose gradient flotation profile, NA functional assay, detection  
232 of multimeric NA, TEM study, as well as desialylation of surface sialic acid on the cells  
233 producing NA-VLPs. The presence of both influenza virions and NA-VLPs in the middle  
234 fractions of the sucrose gradient (Fig. 3a and b-d) indicates that VLPs have a buoyant  
235 density equivalent to native influenza viruses. The enzymatic activity assay showed that NAs  
236 on the particle surface were functionally active and the activity was in proportion to the  
237 amount of protein (Fig. 3f). The morphology of the VLPs was found to resemble influenza  
238 virions. Most of the VLP were spherical although elongated particles were also detected (Fig.  
239 2b & 2f). No morphological difference was observed in NA-VLPs from high pathogenic avian  
240 influenza H5N1, seasonal H1N1, and pandemic swine-origin H1N1 (Supplementary data S1).

241

242 It is conceivable that exosomes or vesicles arising from apoptotic bodies may be  
243 mistaken as VLPs. However, electron microscopy failed to reveal either apoptosis or the  
244 formation of intracellular vesicles (such as the multivesicular bodies of exosomes).  
245 Furthermore, we also monitored for apoptosis in transfected cells by attempting to

246 demonstrate the expression and cleavage of caspase-3 in the course of the experiment. Only  
247 the uncleaved form of caspase-3 could be detected for the first 60 hours post-transfection  
248 suggesting that VLP-producer cells were not undergoing apoptosis (Fig. 4b). Cross-sections  
249 of membrane protrusions such as filopodia (Fig. 2a, arrowhead B) do occur and these may  
250 be mistaken as budding of VLP. However, they can be distinguished from the “typical VLP”  
251 by electron microscopy because VLPs (Fig. 2a, arrowhead A) have a more electron dense  
252 outline which is distinguishable from the cell membrane, except at the budding sites of virus  
253 or VLPs (Fig. 2). Taken together, these data indicate that the particles observed in the EM  
254 are true viral like particles.

255

256 NAs on the particle surface were found to be tetrameric in the influenza virions, which  
257 is important for some of the NA functions. Using the cross-linker DTSSP, multimeric NA were  
258 protected from urea and we were able to detect NA multimeric complexes comparable to that  
259 found on fully infectious H1N1 virions. This result was expected as maturation of the NA has  
260 been described to take place in the endoplasmic reticulum (ER) where the NA-tetramers  
261 form before going through the Golgi network to the assembly site at the plasma membrane  
262 (Saito et al., 1995). Therefore, budding of the NA-VLPs (or virus) from the cell surface will be  
263 expected to incorporate only mature tetrameric NA proteins with similar oligomerization as  
264 would the influenza virus. Failure to detect NA from virions after DTSSP treatment may be  
265 caused by conformational changes of the protein upon conjugation. The cross-linker may  
266 also modify or hide the residues (such as lysine) involved in the antibody binding site. Our  
267 data indicated that NA on the VLP surface was in oligomeric form although the technique  
268 used did not allow us to quantify the ratio between the monomer, dimer, trimer and tetramer  
269 (Fig. 6).

270

271 All three neuraminidases included in the study were able to cleave cell surface sialic  
272 acids in both  $\alpha$ -2,3 and  $\alpha$ -2,6 linkage. As the technique used is qualitative rather than  
273 quantitative, we could not quantify a preference in the cleavage activity to sialic acid with  $\alpha$ -

274 2,3 linkage (Supplementary data S2), as mentioned in previous findings using H1N1 and  
275 H3N2 virus NA (Mochalova *et al.*, 2007; Franca de Barros *et al.*, 2003). We detected a  
276 significant increase of  $\beta$ -Gal-GalNAc in NA-expressing cells by the PNA binding. This  
277 supports the role of NA enzymatic activity during the initial stage of infection by helping the  
278 virus to penetrate through the mucus layer where sialic acid is mainly linked to Gal-GalNAc in  
279  $\alpha$ -2,3 configuration.

280

281 Neuraminidase is an important viral component of influenza viruses and the most  
282 effective anti-influenza drugs in the market are NA inhibitors. Although NA has been studied  
283 for decades, most of the data were obtained from purified virion which requires access to a  
284 high bio-containment laboratory when dealing with highly pathogenic avian influenza viruses.  
285 Purified NA proteins were also widely used for NA study but these isolated NA may not have  
286 the same properties as NA on viral surface. It was in fact reported that substrate specificities  
287 of sialidase activity from purified NA and their original viruses were not identical (Nagai *et al.*,  
288 1995). NA-VLPs produced in our study, though non-infectious, were multivalent, antigenic,  
289 enzymatically active and morphologically similar to native influenza viruses. Therefore they  
290 could be a preferable alternative to recombinant NA peptides or proteins as immunogen to  
291 produce antibodies against neuraminidase or as antigens for ELISA type assays. In addition,  
292 because NAs on the VLPs surface were not only functionally active but also presented in  
293 multimeric form, these VLPs are a useful tool to investigate NA interactions with the host  
294 cells, as well as neuraminidase inhibitor drugs.

295

## 296 **Methods**

297 **Plasmids.** Plasmids pcDNA-HA, pcDNA-NA(H5), and pcDNA-M1 respectively  
298 corresponding to DNA sequences of HA (GI:126361929), NA (GI: 126361907) from influenza  
299 A/Cambodia/JP52a/2005 (H5N1) and M1 (GI: 81975893) from influenza  
300 A/Goose/Guangdong/1/96 (H5N1) were cloned into the mammalian expression vector  
301 pcDNA3.1 (Invitrogen). Both non-tagged and FLAG-tagged HA and NA proteins in the C-

302 terminus were used. For the study of neuraminidase from different viral subtypes, plasmids  
303 pcDNA-NA(H1) and pcDNA-NA(pdmH1) encoding the FLAG-tagged NA of influenza  
304 A/Gansu/Chenguan/1129/2007 (seasonal H1N1; GI:194140557) and A/California/04/2009  
305 (swine origin pandemic H1N1; GI:227977118) were prepared respectively. Enzymatically  
306 inactive pcDNA-NA(E262D) was prepared from pcDNA-NA(H5) by point mutation using  
307 QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer's  
308 instructions and post-sequencing selection of clones based on NA-Star chemoluminescent  
309 assay (Applied Biosystems).

310

311 **Cells and viruses.** HEK-293T and MDCK cells were grown in Dulbecco's modified  
312 Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 %  
313 penicillin-streptomycin at 37 °C with 5 % CO<sub>2</sub>. Influenza A/WSN/33 (H1N1) virus was  
314 propagated in MDCK cells with culture medium supplemented with 0.5 µg.mL<sup>-1</sup> TPCK-treated  
315 trypsin. Highly pathogenic avian influenza (HPAI) A/Hong Kong/483/97 H5N1 virus was  
316 propagated in MDCK in MEM culture medium.

317

318 **Antibodies and Western blotting.** Vaccinated chicken sera containing anti-HA  
319 antibodies (haemagglutination inhibition titer >256) and polyclonal antibodies from rabbit  
320 hyper-immunized with avian Influenza A neuraminidase (Abcam) were used to detect non-  
321 tagged HA and NA proteins respectively. Anti-FLAG monoclonal mouse antibody (Sigma)  
322 was used to detect FLAG-tagged HA and NA. Monoclonal mouse antibody GA2B (Abcam)  
323 was used for the detection of influenza M1 protein. For western blotting, samples were  
324 resolved in 4-12 % SDS-PAGE (Invitrogen), electroblotted to polyvinylidene fluoride  
325 transfer membrane, hybridized with appropriate antibodies and detected with ECL Western  
326 Detection Reagent (Amersham). Protein size was estimated using the Novex® Sharp Pre-  
327 stained Protein Standard (Invitrogen). The fraction of protein released in supernatant (SN)  
328 was expressed as a percentage of overall expressed protein both in lysates and in SN  
329 adjusted to the respective volumes followed by normalization relative to GAPDH

330 housekeeping protein expression detected with anti-GAPDH Mouse Monoclonal (6C5)  
331 antibody (Abcam). Quantification was performed by densitometry on scanned images  
332 obtained using Scion Image Beta 4.0.3 (Scion Corporation). Each quantification was  
333 associated with a calibration curve using a range of BAP-flag protein (Sigma) covering the  
334 range of concentration measured. The exposure times were chosen in order to quantify the  
335 signal intensity within the linear range of the calibration curve below saturation of the signal  
336 as monitored by the image analysis.

337

338 **Detection of VLP production in cells expressing influenza HA, NA and M1**  
339 **proteins.** HEK-293T cells were transfected with different combinations of plasmids using  
340 CalPhos Mammalian Transfection Kit (Clontech) according to the manufacturer's instructions.  
341 The amount of plasmids pcDNA-HA, pcDNA-NA(H5) and pcDNA-M1 in the transfection  
342 mixture was 5 µg each, and empty vector was used to replace the omitted plasmids when  
343 appropriate. At 12 hours post-transfection, the medium was replaced with fresh DMEM-10%  
344 FBS, and exogenous bacterial neuraminidase (*Vibrio cholerae*, Roche; 6.25 mU.mL<sup>-1</sup>) was  
345 added to the medium when appropriate. At 60 hours post-transfection, supernatant was  
346 collected and cell debris were removed by filtration through 0.45 µm filters. Filtered culture  
347 medium was then layered onto a 30 % sucrose-HEPES buffer (2 mM HEPES, 125 mM NaCl,  
348 0.9 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, pH 7.4) cushion and centrifuged at 28,000 rpm for 2.5 hours at  
349 4 °C and the pellet was resuspended in HEPES buffer. Cell lysates were collected at the  
350 same time by the addition of lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.1 %  
351 SDS, 1 % Triton-X, protease inhibitor mixture (Roche Diagnostics) followed by centrifugation  
352 at 13,000 rpm for 10 minutes. Samples were mixed with 4X LDS sample buffer (Invitrogen)  
353 and dithiothreitol (DTT, 100 mM), boiled for 8 minutes and analyzed by western blotting as  
354 described above.

355

356 **H5N1 virion preparation and analysis.** MDCK cells, plated the day before, were  
357 inoculated for 1 h at 37 °C with H5N1 virus at multiplicity of infection of 1. After washing with

358 phosphate buffer saline (PBS), infected cells were incubated at 37 °C overnight. Twenty  
359 hours post-infection, the supernatant was harvested, clarified by centrifugation before  
360 concentration on 100 kDa pore filter Amicon system (Millipore) and finally resuspended in  
361 lysis buffer (50 mM KCl, 1 % NP-40, 25 mM HEPES (pH 7.4), 1 mM DTT, protease inhibitor  
362 mixture (Roche Diagnostics)). Cell lysates were also collected after addition of lysis buffer to  
363 the infected cells. In each sample, loading buffer (prepared from 6X: 62.5 mM Tris-HCl (pH  
364 6.8), 25 % Glycerol, 2 % SDS, 0.01 % bromophenol blue, 5 %  $\beta$ -mercaptoethanol) was  
365 added, boiled for 10 minutes so they can be taken out from the biosafety level 3 laboratory  
366 (BSL-3).

367

368 **NA-VLP and H1N1 virion preparation and analysis.** For NA-VLP production, HEK-  
369 293T, HeLa, and A549 cells were transfected with 10  $\mu$ g of pcDNA-NA(H5), pcDNA-NA(H1)  
370 or pcDNA-NA(pdmH1) as indicated above. Transfection with equal amount of empty vector  
371 was carried out as a mock control. After ultracentrifugation of clarified supernatant (as  
372 described above), the pellet was resuspended in NTE (100 mM NaCl, 10 mM Tris (pH 7.4), 1  
373 mM EDTA), put onto a sucrose gradient (20-60 %) and centrifuged at 35,000 rpm for 2 hours  
374 at 4 °C. Twenty fractions were manually collected from the top to bottom.

375

376 Influenza A/WSN/33 (H1N1) virions were purified from infected MDCK cultures at 72  
377 hours post-infection. The supernatant was harvested and clarified by centrifugation at 4,000  
378 rpm for 15 min. Virions were then pelleted, put onto sucrose gradient (20-60 %) and  
379 centrifuged as described above for VLPs. Recombinant NA proteins purified from transfected  
380 cell lysates by immunoprecipitation using anti-FLAG M2-agarose affinity gel (Sigma), were  
381 layered onto sucrose gradient as above, as additional control.

382

383 All the upper-mentioned sucrose fractions of VLPs, virions and purified proteins were  
384 boiled in LDS loading buffer with DTT, and analyzed by western blotting (see above).  
385 Sialidase activities of the NA-VLPs and purified proteins were measured by NA-Star

386 chemoluminescent assay (Applied Biosystems). Oligomerization of NA on the VLP surface  
387 was studied using the cross-linker 3,3'-dithio-bis(sulfosuccinimidylpropionate) (DTSSP,  
388 Thermo Scientific). NA-VLPs were mixed with DTSSP (5 mM) to stabilize the multimeric NA  
389 complexes, prior to the addition of LDS loading buffer followed by addition of urea (8 M) or  
390 DTT (100 mM) when appropriate. DTSSP pre-treated VLPs were directly analyzed by  
391 western blotting as described above.

392

393 **Electron microscopy (EM).** Transfected HEK-293T cells were fixed with 2.5 %  
394 glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1 % osmium tetroxide.  
395 The fixed cells were then pelleted in 1 % agar, dehydrated in increasing concentration of  
396 ethanol, and embedded in epoxy resin. Ultra-thin sections of the samples were visualized  
397 with Philips EM208s electron microscope after staining with 2 % aqueous uranyl acetate and  
398 Reynold's lead citrate.

399

400 **Kinetics of NA-VLP release.** HEK-293T cells were transfected with pcDNA-NA(H5)  
401 or pcDNA-NA(pdM1) as mentioned above. At 12 hours post-transfection, the culture  
402 medium was replaced with fresh DMEM-10% FBS. Supernatant and cell lysates were  
403 collected at 20, 28, 36 and 60 hours post-transfection as described above. Samples were  
404 boiled with LDS loading buffer and DTT for 8 minutes and were analyzed by western blotting.

405

406 **Effect of neuraminidase enzymatic activities on production of VLP.** A single  
407 amino acid change E262D in the NA is known to abrogate the sialidase activity of the NA  
408 molecule (Huang et al., 2008). HEK-293T cells were transfected with pcDNA-NA(H5) or  
409 pcDNA-NA(E262D), with or without pcDNA-HA. At 12 hours post-transfection, the culture  
410 media were refreshed and sialidase inhibitor (Oseltamivir carboxylate, 50  $\mu$ M) was added to  
411 the medium as indicated. Supernatant and cell lysates were collected at 60 hours post-  
412 transfection and protein levels in VLPs were analyzed by western blotting. Sialidase activities



413 of the supernatant were measured by NA-Star chemoluminescent assay (Applied  
414 Biosystems).

415

416 **Lectin staining for cells surface sialic acid.** Lectin histochemistry analysis was  
417 performed as previously described (Nicholls *et al.*, 2007). Briefly, HEK-293T cell lines stably  
418 or transiently expressing N1 from seasonal H1N1 (HuN1) or pandemic H1N1 (pdmN1) or  
419 avian influenza H5N1 (AvN1) were fixed with 4 % paraformaldehyde (PFA). After washing  
420 with PBS, lectin staining was performed with *Sambucus Nigra* agglutinin (SNA-I, Roche) and  
421 *Maackia amurensis* agglutinin (MAA-II, Vector) to detect  $\alpha$ -2,6 or  $\alpha$ -2,3 linked sialic acid (SA)  
422 respectively. Peanut agglutinin (PNA, Roche) was used to identify the terminal Gal-GalNAc.  
423 Biotin conjugated lectins were used with avidin-peroxidase counterstaining (Vector).

424

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432

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526

527 **Figures legends:**

528

529 **Figure 1. Detection of viral proteins from transfected HEK-293T cells.** (a) Evaluation of  
530 the contribution of different viral structural proteins HA, NA, and M1 from H5N1 expressed in  
531 HEK-293T cells in combinations as indicated and comparison with H5N1 whole virus (b).  
532 Exogenous bacterial NA (exoNA) was added to the medium when indicated. VLPs released  
533 in the culture medium were harvested at 60 hr post-transfection and pelleted through a 30 %  
534 sucrose cushion. Samples were analyzed by SDS-PAGE followed by immunoblotting to  
535 detect different viral proteins together with the lysates of transfected cells. Percentages of  
536 protein released were calculated as fraction detected in the supernatant from the overall  
537 protein detected both in lysates (Lys) and supernatants (SN). Apparent sizes of the HA, M1  
538 and NA were ~27 kDa (HA2), ~28 kDa and ~55 kDa respectively.

539

540 **Figure 2. Transmission Electron Microscopy of cells expressing HA, NA and M1 from**  
541 **A/Cambodia/JP52a/2005 (H5N1).** HEK-293T cells were processed for electron microscopy  
542 at 36 h post-transfection. VLP budding was observed in cell expressing HA without  
543 exogenous NA treatment (a & b) or with the addition of exogenous NA (c) or when NA was  
544 expressed alone (d & f), but not when M1 was expressed alone. (e). Arrowheads : A: VLP; B:  
545 filopodia.

546

547 **Figure 3. Physical and functional characterization of NA-VLPs.** Western blot analysis of  
548 fractions from sucrose gradient from 20 % (fraction 1) to 60 % (fraction 20) sucrose of  
549 A/WSN/33 (H1N1) virions collected from infected MDCK cells (a), from supernatant of HEK-  
550 293T cells transfected with NA plasmid from seasonal H1N1 (b), pandemic H1N1 (c), HPAI  
551 H5N1 (d) or from immuno-purified N1 protein (e). Neuraminidase enzymatic activity of the  
552 corresponding sucrose fractions were tested with NA-Star chemoluminescent assay (f) and  
553 expressed in arbitrary unit (AU) defined as the percentage of the fraction with the highest  
554 signal.

555

556 **Figure 4. Kinetics of N1-VLP release.** (a) Neuraminidase enzymatic activity (expressed in  
557 relative luminescence unit, RLU) from the supernatant of HEK-293T cells transfected with NA  
558 from either pandemic H1N1 (plain line) or H5N1 (broken line) tested with NA-Star  
559 chemoluminescent assay. Experiment performed in triplicate. (b) Evaluation of percentage of  
560 NA released by western blot analysis of supernatant and transfected cell lysates; and  
561 monitoring of Caspase-3 cleavage (uncleaved (#) and cleaved (\*) forms). The percentage of  
562 release was calculated as indicated in Methods section. Lysate from H5N1 virus-infected  
563 cells was used as positive control (Ctl) for cleaved form of caspase-3.

564

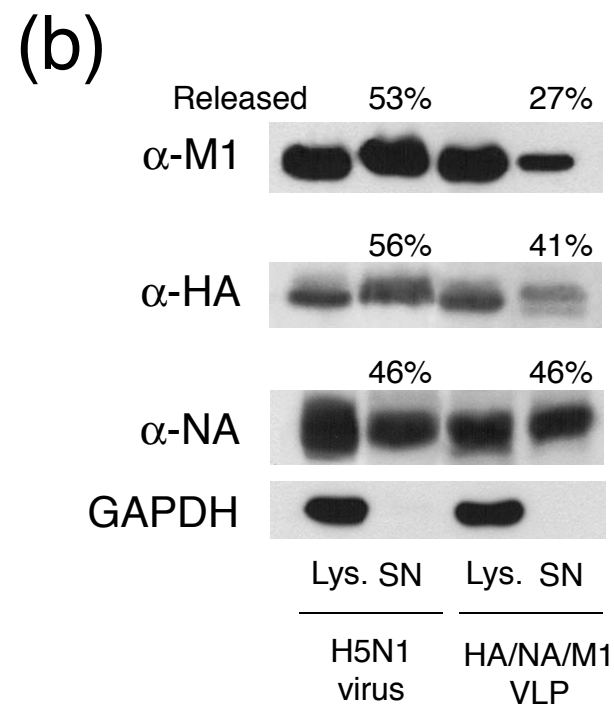
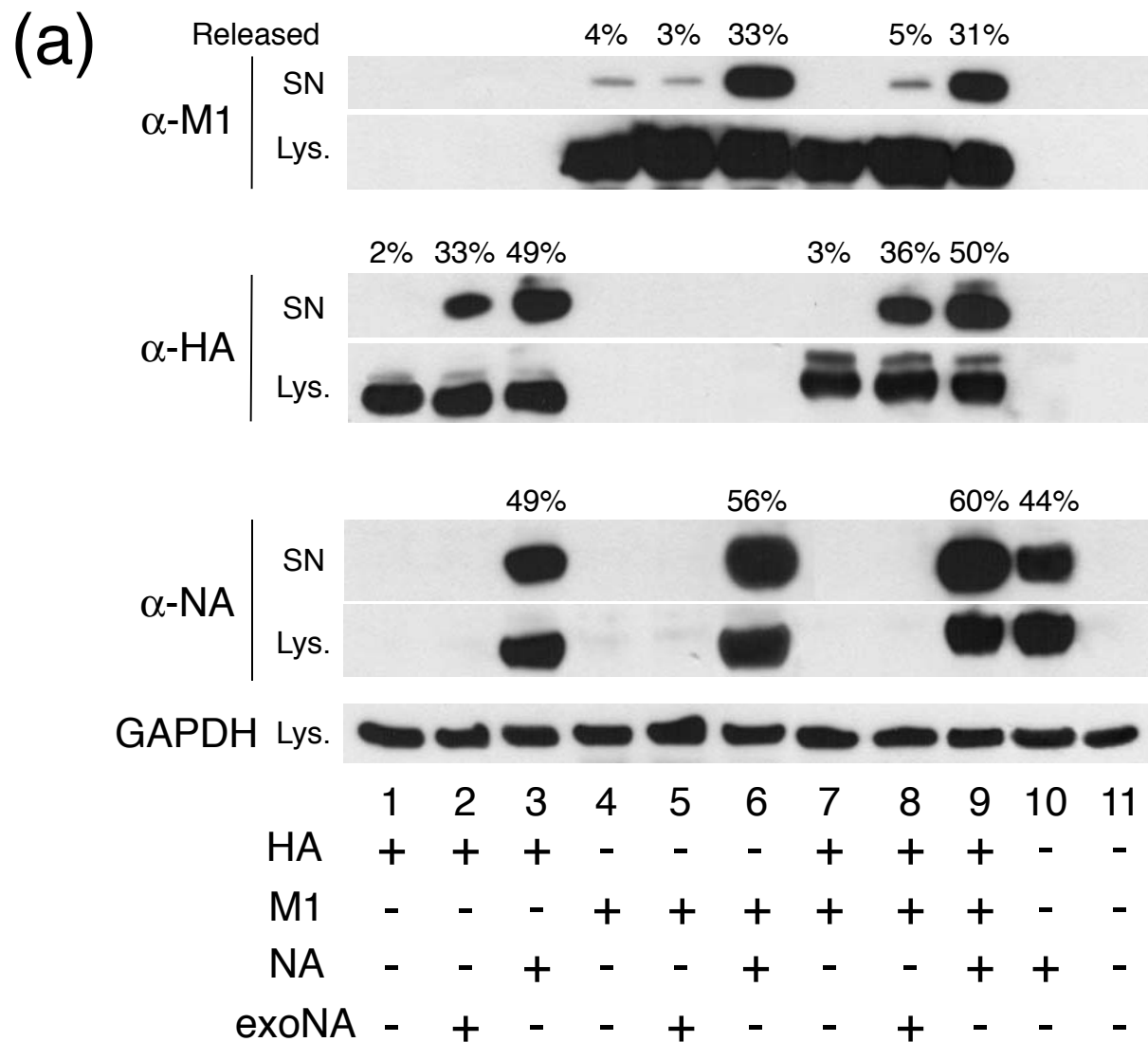
565 **Figure 5. Influence of NA sialidase activity on NA-VLP release.** Neuraminidase  
566 enzymatic activity (expressed in relative luminescence unit, RLU) from the supernatant of  
567 HEK-293T cells transfected with NA (either from H5N1 wild-type (wt), or with mutation  
568 E262D (mut)) alone or co-expressed with HA; in absence or presence of Oseltamivir (Oslt).  
569 Evaluation of percentage of NA released by western blot analysis of supernatant (SN) and  
570 transfected cell lysates (Lys) (2X). Percentage of NA release was calculated as indicated in  
571 Methods section. Bkg: background level.

572

573 **Figure 6. Detection of multimeric NA complexes on VLPs.** N1-VLPs were resolved in 4-  
574 12 % SDS-PAGE after heating in SDS loading buffer (lane 1), and with the addition of urea  
575 or DTT (lanes 2 and 3 respectively). Similar treatments were done on NA-VLPs pre-treated  
576 with cross-linker DTSSP (lanes 4 to 6). Samples from SDS-PAGE were then transfer to  
577 PVDF membrane and immunoblotted with monoclonal anti-FLAG antibodies. H1N1 virions  
578 were included for comparison and NA were detected using anti-NA antibodies. Apparent  
579 sizes of NA monomer, dimer, trimer and tetramer were ~55 kDa, ~110 kDa, ~165 kDa, and  
580 ~220 kDa respectively.

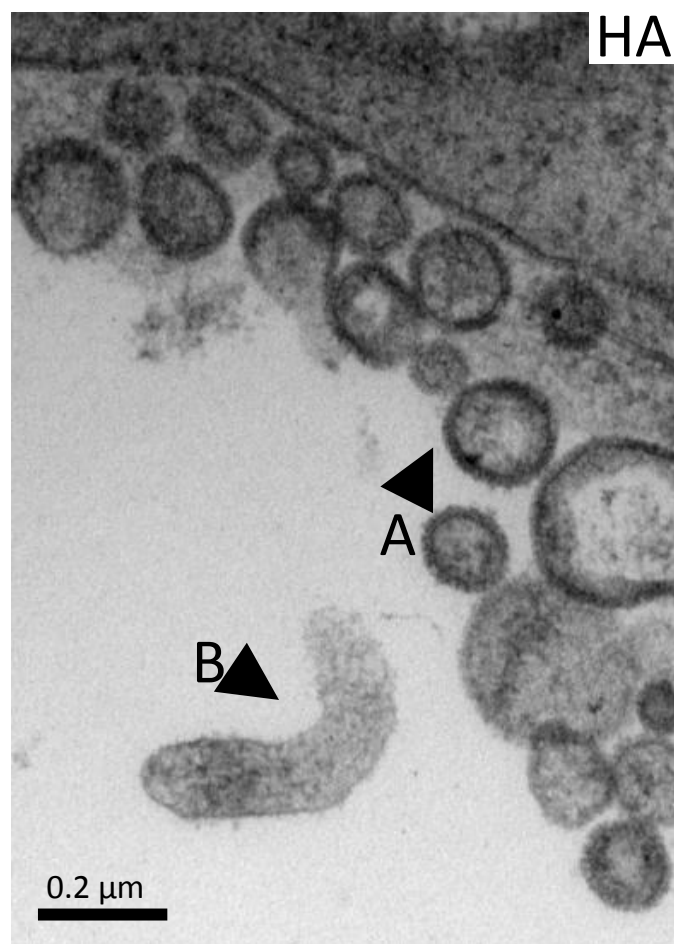
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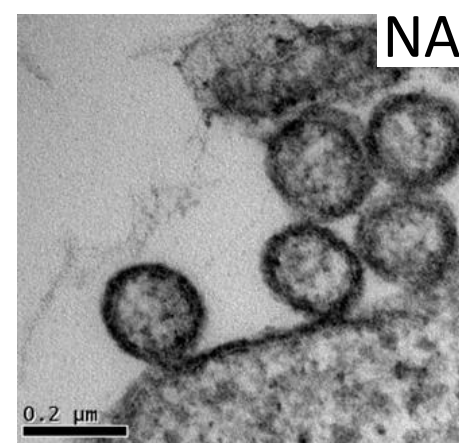




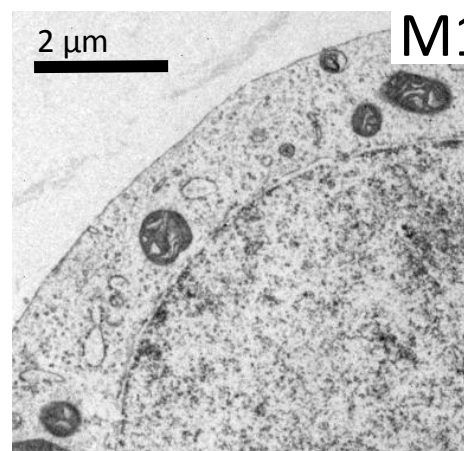
(a)



(b)



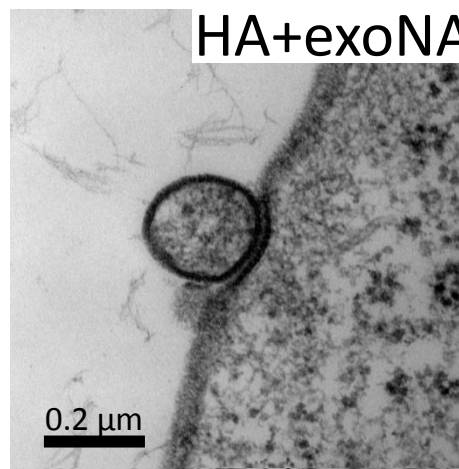
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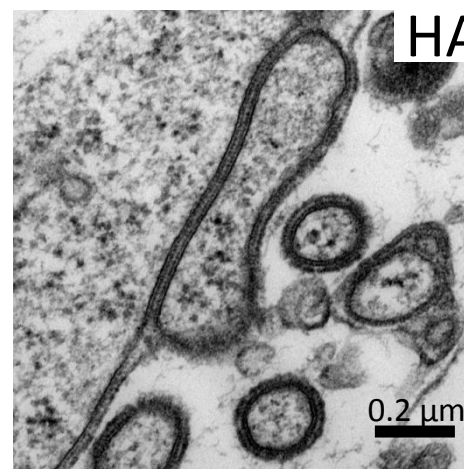
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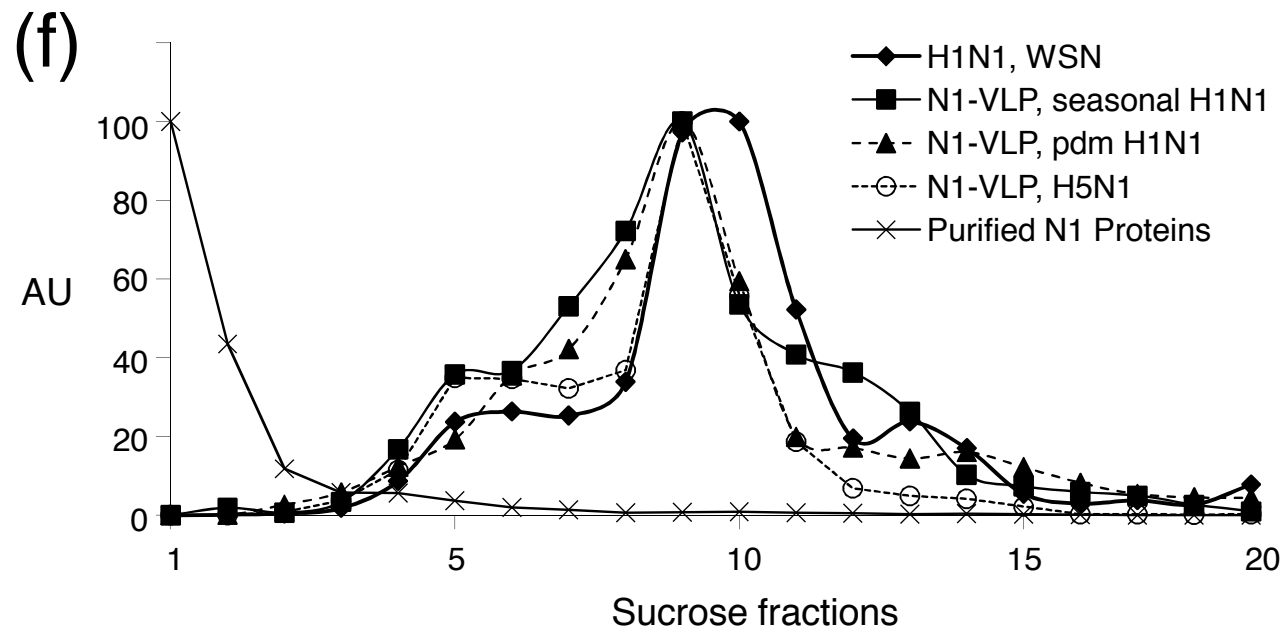
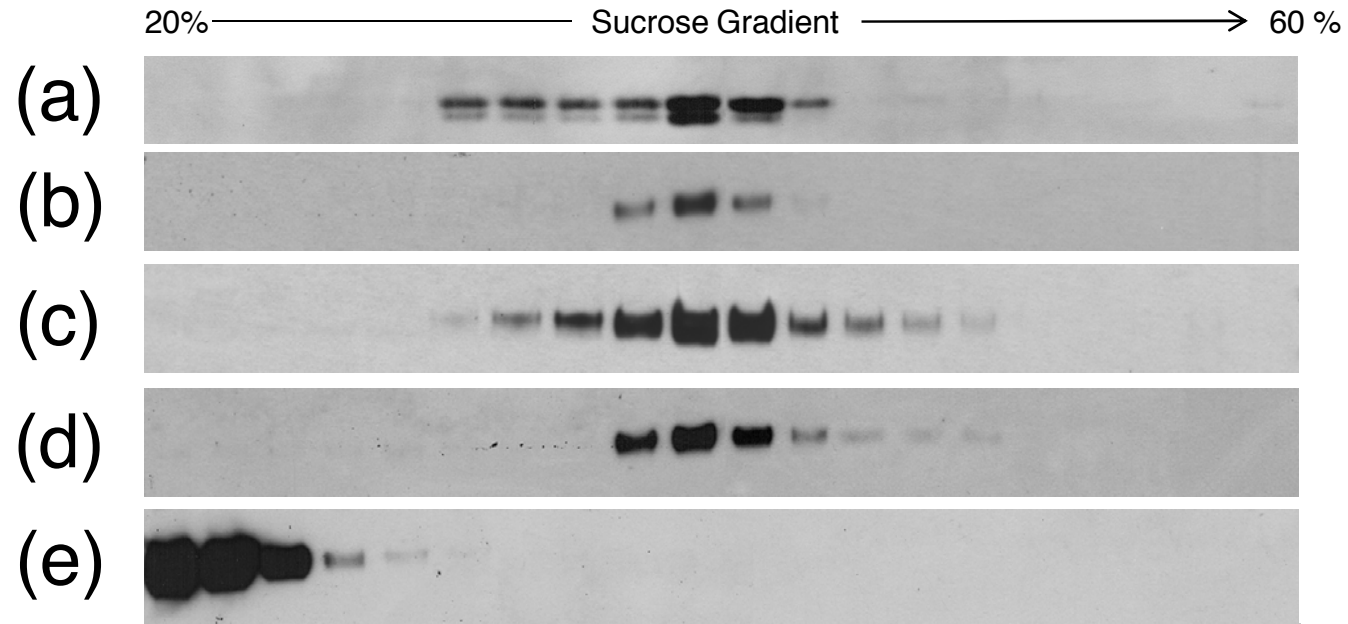


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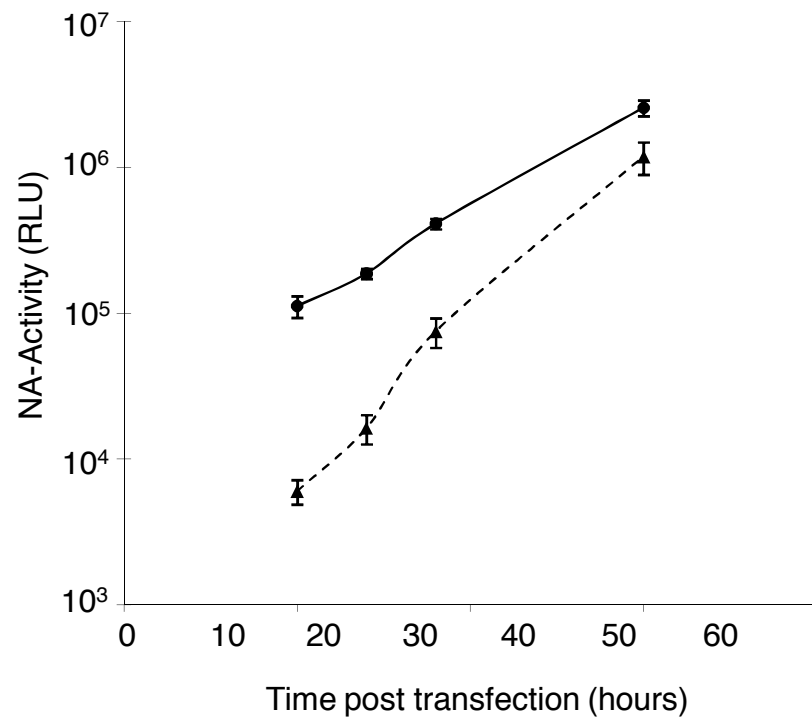


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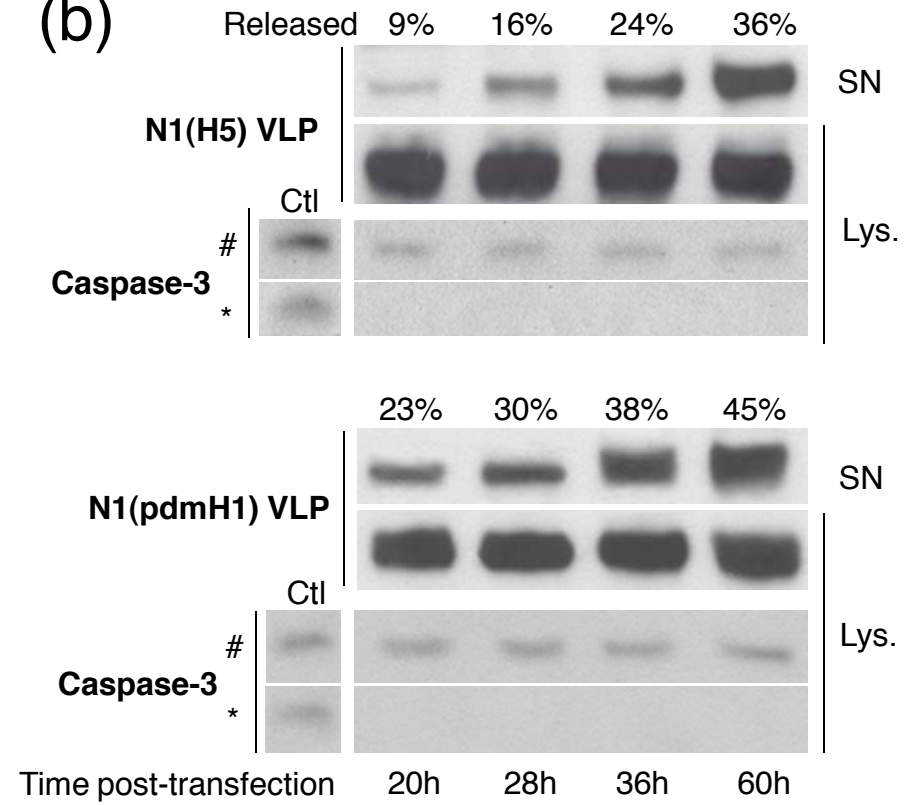


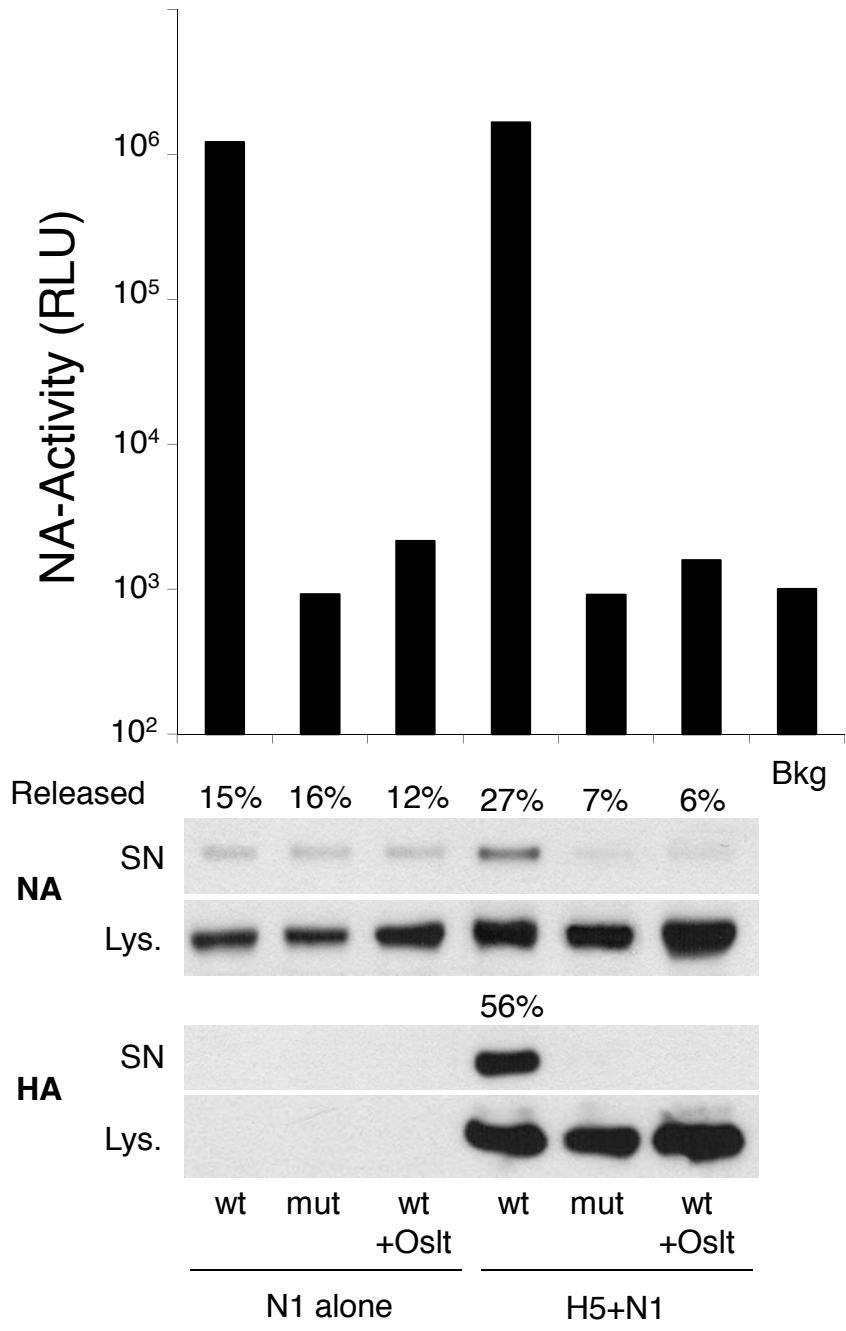


(a)



(b)





SDS	+	+	+	+	+	+	+	+
Urea	-	+	-	-	+	-	-	-
DTT	-	-	+	-	-	+	-	-
DTSSP	-	-	-	+	+	+	-	+

