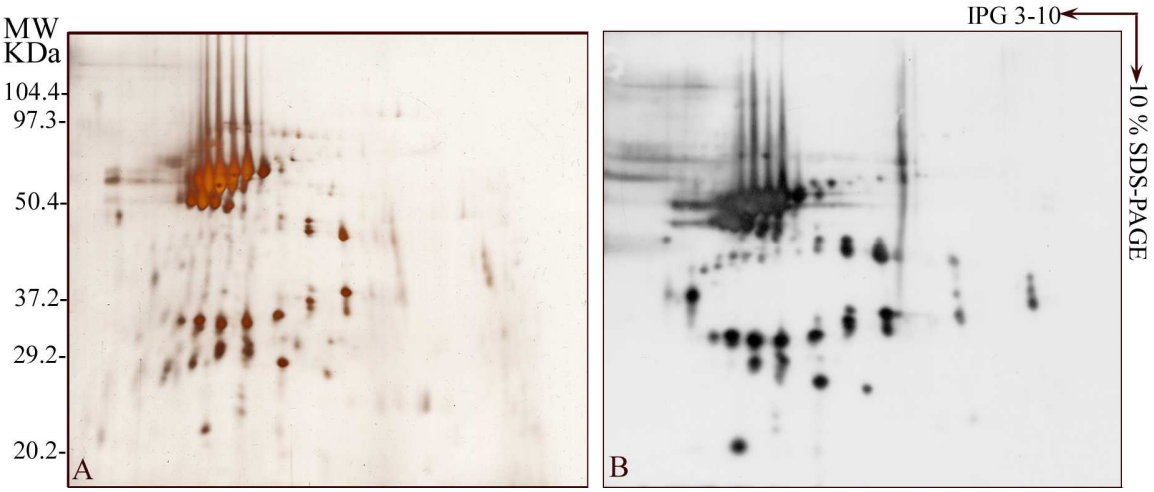
1. SUPPLEMENTARY FIGURES AND LEGENDS



Supplementary Figure 1. 2-D gel of Sputnik protein extract visualized by silver staining (A) or transferred to nitrocellulose and probed with mouse anti-Sputnik antiserum (B). Standard molecular weight markers are indicated on the left.

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                                                P-loop (Walker B)
Virophage+GOS homologs
ORF_13_virophage_Hel          FDNPKPFITSLACALAGEIKLKKIYFCPGKSNAGKSYLIKMLQYCFGDYIGTINGENISYNSK
GOS_2645573                   NGVSTYLLERISKAIAGDFV-KDFNFCLGKSNAGKSILMRMLSLTFQKYVASYNGECMAGTSG
GOS_4362                      EDVADYYILNIARGLAGDAM-KRCLFGIGDGNTGKSAMTSAIKSVAGGYFGTFNANNLVVKKH
GOS_612019                    KNVRNYVMKIFATCLDGTTKREKFYILTGTGGNGKSKLIELFDLAMGDYSKNVSVSLLTKKRA
GOS_2347083                   KGLIEYFQKAAGYSLLGDNREQVMFICHGAGANGKSLLLETIREVVGTYGQTVPVTALMSGK-
GOS_465735                    DDVLEYTLRFLSSCLSGEIREEKFYFWTGSGGNGKSKLVELIDYTLGDYSKSMDVGFLTTKRG
Bacteriophages
phi_adh_CAB52501.1            QELIHFVQKIIGYSLTGSNAEQKMFILYGNGRNGKSVLLNIVKYIFGSYAKTMNATTIMQKRI
P4-like_ABL69094.1            PEVRRFVQRWFALNTTALTGEQKLVFFYGLGANGKSVLVDLIARMFGDYAATARIETLTGSTK
CperCP_ZP_02640204.1          QELINYVQKAVGYSLTGDMSEQCLFMLWGGGANGKSTFVKALEDIMGTYAATIKGETLMEKNG
PSA_CAC85608.1                KELINYIQKAVGYSLSGSTSEQVMFILFGNGRNGKSVFLDIINDIFGSYATNIQPQTIMVKQQ
Lin2587_CAC97814.1            KELINYMQKAVGYSLSGSTSEQVMFILFGNGRNGKSVFLDIINDIFGSYATNIQPQTIMVKQQ
NCLDV
YL207_MIMIV_Q5UQ22.2          KSMREYILTLLSTCLSGTNSEESFYVLTGSGANGKSKLMELLKYTLGDLYKPMDIRLLTEKRS
D5-like_LDV1_NP_078717.1      IELRVFFIRQLASAFIGGNSEKICLFWTGSGNNGKTITQTLMEQMFGPFAVKLNTSVITGKKL
D5_canarypox_AAR83428.1       SENRELYEQILSSCLMGT-TKQCIFFFYGETATGKSTTKKLLKSVMHNMFLETGQVILT-EQM
Archaeal plasmids
primpol_Aamb_CAA12526.1       KWITLF--EIIGYTLYPATKIKLAFMLLGPRDSGKSTFLQLLKKILGKHN----TVSIRVKEL
primpol_Sisl_YP_001569030.1   KWILLF--QIIGYTLYPGIKFRKAFMLVGEGKNGKSSFINLVKKVLGDYAVSISPRELFDPRN
                                          .   .  *    **      .              :

                                                Mg-binding (Walker B)
Virophage+GOS homologs
ORF_13_virophage_Hel          -DSRDEAAKYRWAY-LLANTRIVMSSEI-----SMKKSIDGNMIKKFASAG--DKIVGRKHC-
GOS_2645573                   -EDNAKNYR--WAL-ALRYARLIGSSEI-----EMGITLNGNKIKKG-TGH--DEMVGREHG-
GOS_4362                      -ANPDDAQALRWVM-LLANKRIIASNEL-----EPDVEINGSVLKKL-SSGGKDDIVARKHG-
GOS_612019                    -DSNAAQPE--LAV--TKGRRVIKFQEA-----EENSKLNVGLMKEL-TGG--DKVVCRGLF-
GOS_2347083                   SNSGGANPE--IAR--LRGVRFALASET-----EKGQRWSANRIKQL-TGG--DTVAARALY-
GOS_465735                    -SSSAASPE--LEN--IKNARFVSMSEP-----EKTDTVYIGKLKQM-TGG--DKMTSRGLF-
Bacteriophages
phi_adh_CAB52501.1            GSSQGATSD--IAR--LEGARLVVSSEA-----NEGDRLDESLVKQM-TGG--DTLVARYQY-
P4-like_ABL69094.1            KDGSAATPD--LVP--LMLARMVRTSEP-----EEGEKLREGLIKQL-TGG--EPINVRPNF-
CperCP_ZP_02640204.1          --QDGARGD--LAR--LTNKRVVIASEL-----QEGQVFNEPLLKVL-SAG--ETLPVRFMY-
PSA_CAC85608.1                --SSNANSD--IAR--LHGARFVTTTEP-----NEGVRLDEGLVKQL-TGG--DKVTARHLY-
Lin2587_CAC97814.1            --SSNANSD--IAR--LHGARFVTTTEP-----NEGVRLDEGLVKQL-TGG--DKVTARHLY-
NCLDV
YL207_MIMIV_Q5UQ22.2          -SSSSASPE--LAD--KKGIRACPFDEP-----KASDEINTGFMKIF-TGG--DTITARALY-
D5-like_LDV1_NP_078717.1      -PTGQANPE--LVR-TGGGVRWAVMEEP-----DSDERINAGILKSL-TGN--DTFWARDLYC
D5_canarypox_AAR83428.1       --DKGPNPF--IAN--MHLKRAVFCSELPDFSCNTSKKIRSDNIKKL-T-E--PCIVGRPCY-
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Archaeal plasmids
primpol_Aamb_CAA12526.1       -FDSNNRFV--MGY--LFHKLANLTAET-----KEYTINDIDRFKTL-TGG--DQVTSDVKF-
primpol_Sisl_YP_001569030.1   ------RFI--VGN--LYHKLANAVAES-----KDYSIDDMDRVKRL-TGD--DWITADVKF-
                                                *      .         .*   :


                                        Sensor I motif
Virophage+GOS homologs
ORF_13_virophage_Hel          --ESEISFTPNFTIFCMFNDIPEIEPHD-EAVSNRLVYHEFPYVFVKEEELNEK-PYNKLK--
GOS_2645573                   --GAETSFIPHFSMFLFANDLPKIKPID-DGTKNRCRVVSYEKKFGDTYIEDEQ---------
GOS_4362                      --GYETEYKISFLPIIFANDLDKITPMD-DAIVNRVRAIPYEKKYVEKPKNNN---------Y
GOS_612019                    --QDPIEFKPQFTPFFICNDKPELPPHD-DGTWRRVRIIDFPSKFIPAEQNPDPAK------N
GOS_2347083                   --KDVSEFKSKATIWVACNHKPEVDAAD-TAMWRRMRLVPFLRVFKPEE--------------
GOS_465735                    --KGTTQFKPQFKIVLMCNDLPQLGGND-GGIWRRIEVLKFISKFTNNGKSVNE------DKH
Bacteriophages
phi_adh_CAB52501.1            --GKDFEFDPVFKLFMATNHKPKIYGTD-EGIWRRLVIIPFTHTVKKEN--------------
P4-like_ABL69094.1            --GEQIEVTPKFKITIQGNYRPEVRGRD-DGIWRRLLIVPFDVTIPPKE--------------
CperCP_ZP_02640204.1          --QEEFMLKPKFKLWIMTNKKPKVKGND-HGIWRRWRMIPFKYKFTEKE--------------
PSA_CAC85608.1                --KAEFEFTPEFKIWMATNHKPIIRGRD-DGIWRRLHLVPFTVKIPDEK--------------
Lin2587_CAC97814.1            --KDEFEFTPEFKIWMATNHKPIIRGRD-DGIWRRLHLVPFTVKIPDEK--------------
NCLDV
YL207_MIMIV_Q5UQ22.2          --KEPIYFKPQFKPFLLCNELPTIKSDD-DGTWRRLKVIPFLSKFIKHSEATKKMKKEGLPKN
D5-like_LDV1_NP_078717.1      TGKDTKEIIPMFKLHVICNNLPEIKYAD-QAVWNRVRVIPFESVFKLAEECPDT-YKERLNQK
D5_canarypox_AAR83428.1       --SNKINNRNHATIIIDTNYKPVFDKVD-NAIMRRIALVNFKTHFTNSRKKVYNTKYDFIK--
Archaeal plasmids
primpol_Aamb_CAA12526.1       --NGPITFTPYAKIIIASNKLPNVSDKNDMAFWRRWLIIEFPNTFPNDD--------------
primpol_Sisl_YP_001569030.1   --KDPITFKSVAKLIIASNNMPHVRDTNDRAFWHRWVIVEFPHQFKDND--------------
                                        *    .   :  .   *     :

Virophage+GOS homologs
ORF_13_virophage_Hel          ----DEDLD-SKYQTKDFASGFIHILLDAYKNYLENG-LPE-FDNEVKEKWTAQTKQIDKVTS
GOS_2645573                   -EVIDYKFE-DKMKNPLWINAFCKLIIDNYKQ---DN-VS--VPNLVINDFDEW-TEGDNFKE
GOS_4362                      ELKIDPNFD-EEIQTPKFRGAFMLLLMKAYKKFMKNK-RVENEPEQIRRAFVSTFGKVEEYVD
GOS_612019                    EFPIDYELS-EKIKE--WGEAFLWLLVEHYKEYRKTG-IV--EPSKVMEYTNHYRKKIDIYNT
GOS_2347083                   ---QDPNLS-AKLAE--ERDGILAWMLQGLAMYREQG-LT--EPDAVVKATARYRDEMDSVKR
GOS_465735                    QYCADEQLS-AKLEQ--WKLLFMIMLLKKYEEYDKTG-TL--PPKEVKEETKCYQNSNDIISN
Bacteriophages
phi_adh_CAB52501.1            ---VDKKLE-DKLKA--ESMGILKWAIEGAMMWQSEG-LN--PPDVIQNAGNEYRKEMDVIEA
P4-like_ABL69094.1            ---RDPDLGA-KLWE--ERSGILNWLIEGLIDYLEGG-LQ--EPPAVLSATNEYREESDPLGF
CperCP_ZP_02640204.1          ---KDPNFYEEKLKP--ELEGILLWAITGYQMWKEQG-FE--APKEVMEAVEDYKMDMDQVAR
PSA_CAC85608.1                ---VDKQLK-YKLRR--ELTGILNWAVEGFLKWQREG-LG--MPGAVENASSEYKSEMDVITA
Lin2587_CAC97814.1            ---VDKQLK-YKLRS--ELTGILNWAVEGFLKWQKEG-LG--MPKAVENASSEYKSEMDVITA
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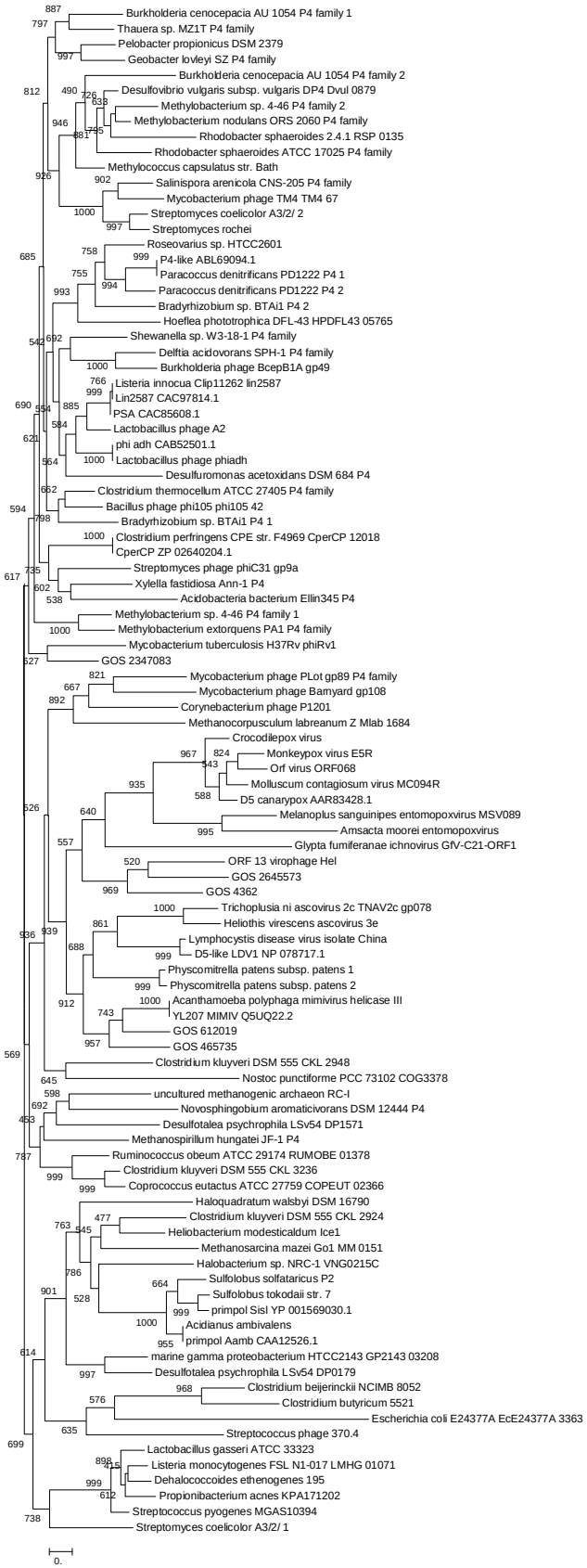
NCLDV
YL207_MIMIV_Q5UQ22.2          HFWADTSLS-EKLPD--WKQGFMCLLLKYFRKYRKHG-LI--HPKLVTQHTVEYRKKCDVFQD
D5-like_LDV1_NP_078717.1      IFPVDLKFS-EKLS--KLIEPLAYYLIYYWLNMDRLN-YN--PPTKVLNATKEYQNDNDIYKQ
D5_canarypox_AAR83428.1       --PLNEGLDS-KIQSNYFRYAFLKILLGWYQKYHVPNLTILPTPDKIPDF--KFRLKIDAL--
Archaeal plasmids
primpol_Aamb_CAA12526.1       -----NWFRQTFTEE--EIEGILTVSILAFARVIINGKFD--YQQTPEEVRGLWLNNIDSVWS
primpol_Sisl_YP_001569030.1   -----SWFDKTFTEE--EINGIITTAIASISRAFAQGHVD--FEQSEEEVMGIWLSHIDSVYN
                                                                                    :

Virophage+GOS homologs
ORF_13_virophage_Hel          IINEYYEV---T----NNVK---DFVPLNEILK--FKEQHKDL
GOS_2645573                   IFENEFNICNYK----PNDDEYKNNFITNQRFS--EWRKDKNL
GOS_4362                      TFKNDFEF---T----DNEN----DFVESSIMI--EWIKQNKL
GOS_612019                    FVNETIV---QE----INAR--LYLNDLYKVYR--DWHKENYA
GOS_2347083                   FLETQAE---VV----SDGR--TPLSRMKEAYQ--HWCRDEGL
GOS_465735                    WVDDCLS---EC----D-DF--TPFELLYDAWE--DYCDDEGI
Bacteriophages
phi_adh_CAB52501.1            FIDECCV---TN----DSYK--VKLPTYLDAYK--NWANETNN
P4-like_ABL69094.1            FLESCCD---VSG--QPEDS--ETVKDLVQAFQ--FWQDEQGG
CperCP_ZP_02640204.1          FIEDCCF---IR----DDAE--CTGSAMYDEYL--NWCINEGE
PSA_CAC85608.1                FIEDCCD---VR----EGEK--VNAKKMYETYH--EWAKENGQ
Lin2587_CAC97814.1            FIEDCCD---VR----EGEK--VNAKKMYETYH--EWAKENGQ
NCLDV
YL207_MIMIV_Q5UQ22.2          FIGDYLV---RV----DNTKKGISVMDLYQNMR--EWYKSNYT
D5-like_LDV1_NP_078717.1      FIDNNLIK---Q----DNII--LTERLLYIRYK--DWLAETHP
D5_canarypox_AAR83428.1       IIPSST----------------THIKYIKELSKLGYIIDEDGL
Archaeal plasmids
primpol_Aamb_CAA12526.1       FIKTYVEKGIITLDPRNADL-WVPRKELYNLYK--EYCLDNGF
primpol_Sisl_YP_001569030.1   FIKTYVEKGTIRLDPKNGDL-WVPKDQLYNLYQ-—NYCIEQGF
```

Supplementary Figure 2. Alignment of the SF3 helicase (C-terminal) domain of ORF 13.



Supplementary Figure 3. Phylogenetic tree of the SF3 helicase (C-terminal) domain of ORF 13

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                                                  Putative primase catalytic motif
Virophage+GOS homologs
ORF_13_virophage_N  DTGRVYAEK-------GRSLQSFKKAIRAFI-NNGINLDIDMKNSHPTLITQYCKKNKI
GOS_8943382         EMGRLYAEK-------SLSLQNFSKPIRHTL-AHDSKIDIDIVNCHPVLLSQYCHKKGI
GOS_425601          TYGRLFAQN--------PSLSALPREIRNSL-AHGQYYDIDMKNCHPSLLRQYCYKNGI
GOS_5897846         LFGRLYPKRERGGS--TPSLQGLKRDVRKAL-AHDQYTDVDMVNAHPVILSQLFLKLGL
GOS_2251034         RRGRLYPKR------GASSVQGLKRDVRKAL-THDRYTDIDMVNAHPCILSQLFKKHNL
GOS_92763           KGGRLYCG---------NSIQGLPKFIRGFL-MK-HTTDIDAKNCHPTILRYLCKIHNI
GOS_386275          VNGVKYHQRNGMGRFYGKTFTGLTKKMKHTLFSYANMVDIDQHKGHPRIAVGLGDLNGT
GOS_3337959         AWGRVFPIK-------ALGCTSFAKKTRNTL-IKDFYLDFDLSNAQPEILRNICLANNI
                      * ::               : :  :  :      *.*  : :* ::
Virophage+GOS homologs
ORF_13_virophage_N  LCPFLDDYVRRREKRLEDVMV-----------FHKISRDQAKELILRLCYLGSYKIPND
GOS_8943382         ICDKLDHYNEHREMLLSELME-----------CCKCSRGEAKRLVLMLMYLSTVGEACV
GOS_425601          RCDTLDSYVKNRDEVLSKICT-----------ENNVQRDDAKQEILTIMNGGKGKWQIS
GOS_5897846         ACPALERYVAEREAVLAETGL--------------GRDEAKQAFITLMYGGERK-----
GOS_2251034         ECPLLDEYIANREEHLENVVGI--------------IDDTVTALLEDTSKNR-------
GOS_92763           ECPKLDFYIENRDIIINMGTAK--------------KDDYLKSINDGNINKK-------
GOS_386275          DFETMKYYIENDEKVFRDMADW-YGVDIDDTEKGSQNKDRLKWFFNMSIYGGGYKKEEW
GOS_3337959         PCEIITKYCVDREEIIADIIKKSNNKVNRSLVKSLMIRLSFCGTFKNWLRKESIEP--F
                       :  *   ::  :
Virophage+GOS homologs
ORF_13_virophage_N  DGTSYKPK----------------------------KTLEFLEKFKEEAEIIADRIAK
GOS_8943382         KIGISV------------------------------PPPEWLDELEEHLKQLAEMIVA
GOS_425601          K-----------------------------------VPGTFVYDFKQEIRTIHDHVCR
GOS_5897846         ----------------------------------------DPTPFMAEFREEFLTNA
GOS_2251034         ----------------------------------------KVKSEHTRKSSSLRRD
GOS_92763           -----------------------------------IKDIFFNDFDKEIKQIHKKIMV
GOS_386275          MKGDQDNSWLNGIINPCEKDRASGYEPLELKTHEQMPFMKAFKAECNLLKELIWKNNLL
GOS_3337959         -----------------------------------DEPVIIADYCAEVRSITQTIIK

Virophage+GOS homologs
ORF_13_virophage_N  -----KEK--ELYAKIKD---NDDCKNKKAVILSV-LAQQLEHSCLMEMY
GOS_8943382         -----LNP--EIFKKVS-TSRSKEHTNKKASCVSY-VLQNIENDLICNAR
GOS_425601          -----LNP--DEFKKVQ----RRKDFNKEGTMMNI-ILCKLEHELLMHSV
GOS_5897846         TAVLASEA--YARYRTL---AEAKKPANVLGCGIS-FVAQDLERQLVCCA
GOS_2251034         EAKTQFLRVMYGGRPDTLSIETRDKESLYIDWMPPEFLRLFHKEFRQNST
GOS_92763           -----IEEYNDIIMSV---PKDKKEYNWNGSSLNR-ILCMYENNILKEVI
GOS_386275          MKE--VLSKDEEYVSK---PEYRKKNCLVSYFMQV-IENDALFHAYKYLK
GOS_3337959         -----ENP--SMFKTI---SRIKKEKGESNINGAF-LSTYLQEWELRLVE
```

Supplementary Figure 4. Alignment of the putative primase (N-terminal) domain of Sputnik ORF 13

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                                    P-loop (Walker A)
virophage+GOS homologs
ORF3_virophage_FtsK-like      MYREVIYIAGQSGSGKSTYAAQYIYHYKKLFP-----KNKVFVFSRLKMAEILVSLGCI-EIP--
GOS_8413292                   SEREILYITGASGSGKSTYTRLYCEQYKKKYP-----KNPIILFSSLPEDESLDSIKPR-RFK--
GOS_6388064                   VARDILYVVGASGSGKSYFTRQFADQYRKLYP-----KRKIFLISSLTEDNSIDKIKDLKRIK--
GOS_6158                      VARH-CLVSAPSGAGKSFWTGKYAKEYTKLFK-----HNELFLFSAVDEDKALDNLKPV-RVM--
GOS_8407369                   EQVDSLFVCGPTGCGKSSFIRDYCIMFNNKFP-----NAKIYLFSSKREDEVLDKLGYIQRVE--
NCLDV
A32-like_irido_AAY58049.1     MGGMKLIVLGKPQRGKSVLIKSIIAAKRHIIP------AAVVISGSEEANHFYSKL---------
A32-like_asco_YP_762465.1     QGGSKIAFVGKPGTGKSVMMRYIMYTKRKMIP------VAVVMSGTESSTGFYSRI---------
A32-like_phycodna_ABY27879.1  SDDRVCVFIGKRNTGKSTLVKDIMYHKKHI-P------AGIVLSGTEEGNHFYGEF---------
A32-like_Mimi_AAV50705.1      VIDPSIVMIAKRGSGKSWIVRDVMYHYRHL-P------CGVVIAPTDRMNSFYKYF---------
A32-like_swinepox_AAL69857.1  KSPFRLALVGGSGSGKTMYLLSLFSTLIDKYKHIFLFTPVYNEAYDSYIW--PDHVNKVTTSEEL
archaeal viruses
ORF_STSV1_CAH04252.1          TSFSIFVIEGEQGAGKSSMALQILAGIYGYW-----PDDPLLNFQFALYFNIIDPL-QLRDFI--
ORF_SSSV2_AAQ73250.1          VGFVSAVIFGKQGSGKTTYAFKVSRDVFWKLNNLSTKDDAWQYVQNSYFFELPDALSKIQDAID-
ORF_ATTV_CAI59904.1           GLFNQFIVEGQQGAGKSSFALWVARAIYGNWHD------------ALKHVVIDPF---------
                                     . .    **:                                  .


                                                                  Mg-binding (Walker B)
virophage+GOS homologs
ORF3_virophage_ftsK-like      -----IDDELQD----MDAIRDI----------------------KDALCLFDDID--TIKEKHL
GOS_8413292                   -----IDDRLLDEPITTDNIGIF----------------------QDSCIIFDDID--VLTNKKH
GOS_6388064                   -----LTPEFLMDDIQA---EDF----------------------KDSLVIFDDCE--ALMDKRM
GOS_6158                      -----IDSELITDPIQADEL-------------------------HDSLVIFDDTD--SISNPLL
GOS_8407369                   -----IDDDILHNPYTLQQISELS---------------------EPSLCVFDDIE--DFSNKKI
NCLDV
A32-like_irido_AAY58049.1     -----LPNCFVYNKFDADIITRVKQRQ--LALKNVDPEH------SWLMLIFDDCMD----NAKM
A32-like_asco_YP_762465.1     -----VPDAYIHNDCDQMALENFKERQLEARKRCVN---------PWALLVLDDCST----NKKN
A32-like_phycodna_ABY27879.1  -----IPDLFVYGEYDRDAIERVISRQRKIVGTKGKNPY------NGAFMLLDDCMY----DSKF
A32-like_Mimi_AAV50705.1      -----FPDLFIHYEITEAILKNILLRQQMIIDKQKQKKKQGLKIDPSGILIMDDCLS----QKKN
A32-like_swinepox_AAL69857.1  EYSLVTTKQKIEKYIESKGTKNA----------------------DMFLIILDDMGDKQTRSSCL
archaeal viruses
ORF_STSV1_CAH04252.1          ---------------LFLERNEL----------------------RVPAILLDDAQ--VFFGSHT
ORF_SSSV2_AAQ73250.1          --------------------NDY----------------------RIPLLIFDDAG--IWLSKYV
ORF_ATTV_CAI59904.1           ---------QLRQIILVAEANSI----------------------TIPLIVVDDAG--LFFSKGL
                                                                             :.**
```

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                                        Sensor I motif
virophage+GOS homologs
ORF3_virophage_ftsK-like      ---RN-TVYDIQNDILETGRHNNIYIIVTSHL
GOS_8413292                   ---RQ-AVLDIANCVLEIGRHFKITAIFTNHL
GOS_6388064                   ---KL-KVQGILNQLLTIGRHHNISVCELRHN
GOS_6158                      ---LS-AVHHLKERLLEVGRHYNISTIQCNHM
GOS_8407369                   ---NT-EIARLSNEILRNGRSYKIYLITVNHQ
NCLDV
A32-like_irido_AAY58049.1     ----F--NHEAVMDLFKNGRHWNVLVIIASQY
A32-like_asco_YP_762465.1     ------FTTKIQEDLFKNGRHYKMLYLVGVQY
A32-like_phycodna_ABY27879.1  ----L--KDTCIRQCFMNGRHYNIFFMLTMQY
A32-like_Mimi_AAV50705.1      ---WS--KIQEITEILMNGRHYRLTYVLTMQT
A32-like_swinepox_AAL69857.1  ------------LDLLNHGRHLNISIILLCQT
archaeal viruses
ORF_STSV1_CAH04252.1          YWSRR-NRYQVANDLLTLLRPRVSSIIITMPT
ORF_SSSV2_AAQ73250.1          WYE---DYMKTFYKIYALIRTRVSAVIFTTPS
ORF_ATTV_CAI59904.1           AYRRQTGRMQTIQRLLQVIRTAVSNIILTTPN
                                                *
```

Supplementary Figure 5. Alignment of the FtsK/A32-like ATPase (ORF 3)

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                                                                  Zn-ribbon


virophage_ORF_14  KKYYEENKPKIKKYYSK-----------------------------KIECPICGALYTRSNVTNHKKSQKHIKA
GOS_3284690       KKYYEKNKKIILEKGKKHYEKNKEKILEGHKKYREKNKEKILEKGKEKVECP-CGSVVRKDNLPKHKKTQKHQNY
GOS_6504063       KEYYQKNKEKLKEKKKQYYENNKESKKKYDKDYYENKKDKL----KEKIECPICNSIVRKAGLSRHKKTKKCMNA
GOS_1049          RQYAQDHKEELAEWRKQYQLDHKEELTEYQKQYREANREALYKRQLEKVQCERCSAFIARSTFSRHQKTKKCMNA
                  ::* :.:*  : :  .:                              *::*  *.:.  :  ...*:*::*  :
```

Supplementary Figure 6. Alignment of the Zn-ribbon containing protein (ORF 14)

```
ORF_17_Virophage                    MDLRLQQKILKYQQVRKYYV----EDGYTIDEACKKVKINKTTFYNYRKLL------KEN
GOS_7101084                         ------MKASKFSDVQKAFVSKQAEDGVPVAEVCRKAGISQATFFNWKKKYGGMLPNEMR
GOS_9512229                         ------MKASKFSDVQKAFVLKQAEDGVPVAEVCRKAGISQATFFNWKKKYSGMLPNEMR
GOS_9604835                         ------MKASKFSDAQKAFVLKQAEDGVPVAELCRKAGISQATFFNWKKKYGGMLPNEMR
GOS_1672193                         ------MKASKFSDAQKAFVLKQAEDGVPVAEVCRKAGISQATFFNWKKKYGGMLPNEMR
Sagittula_stellata_EBA07908.1       ------MKASKFSDAQKAFIIKQGEEGTPVAEICRKAGISQATYFNWKKKYAGLLPTEMK
Silicibacter_sp.TM1040_ABF65701.    ------MKASKFTDAQKAFIIKQAEDGTSVAEVCRKAGISTATFFNWKKKYAGLMPSEMK
Agrobacterium_tumefaciens_AAK888    ------MKKQRFTEEQIIGVLREQEAGAKAADLCRKHGISEATFYNWKAKYGGMEVSEAK
Escherichia_coli_ZP_00926473.1      ------MRKARFTDHQIIAVLKSVEAGRTVKDVCREAGISEASYYNWKAKFGGMEASDIK
                                           .  .: : .   :    * *    : *.:  *. ::::*:.         . .

ORF_17_Virophage                    NLLEIVETNNNINNILSSEVLNKKKIQSKKTTPKKKYN
GOS_7101084                         RLKQIEDENLRLKSIVADLSLDKEMLQD---VIKRKL-
GOS_9512229                         RLKQLEDENRRLKSIVADLSLDKEMLQD---VIKRKL-
GOS_9604835                         RLKQLEDENRRLKSIVADLSLDKEMLQD---VIKRKL-
GOS_1672193                         RLKQLEDENRRLKSIVADLSLDKEMLQD---VIKRKL-
Sagittula_stellata_EBA07908.1       RLKQLEDENGRLKKIVADLTLDREMLQD---VIRRKI-
Silicibacter_sp.TM1040_ABF65701.    RLRELEQENARLKKIVADLALDKEMLQD---VIKRKL-
Agrobacterium_tumefaciens_AAK888    RLKALEDENAKLKKLLAEQMLDVAALRE---LLSKKW-
Escherichia_coli_ZP_00926473.1      KMKDLEDENRRLKQMFADLSLECRALKD---VIEKKL-
                                    .:  : : * .::.:.:.  *:   :..      .*
```

Supplementary Figure 7. Alignment of the putative IS transposase DNA-binding subunit (ORF 17)

```
ORF_MAEO_YP_001         M-----------------------------------------------DNKKK--------
ORF_MJ_NP_24775         MMIWDWNLSKPS----------------------------------ESIKKH--------
ORF_TK_YP_18251         M-------------------------------------------------VK--------
ORF_ATTV_YP_319         MDKDELKEIIKNLPDNSPKILEYLKLAKEKGWKDIVNMIAQKLGLEEEEKKKTDETDVLK
ORF_STSV1_YP_07         M-----------------------------------------------------------
ORF_10_virophag         MPKYTDDDIFDD-------------------------------------------------
                        *

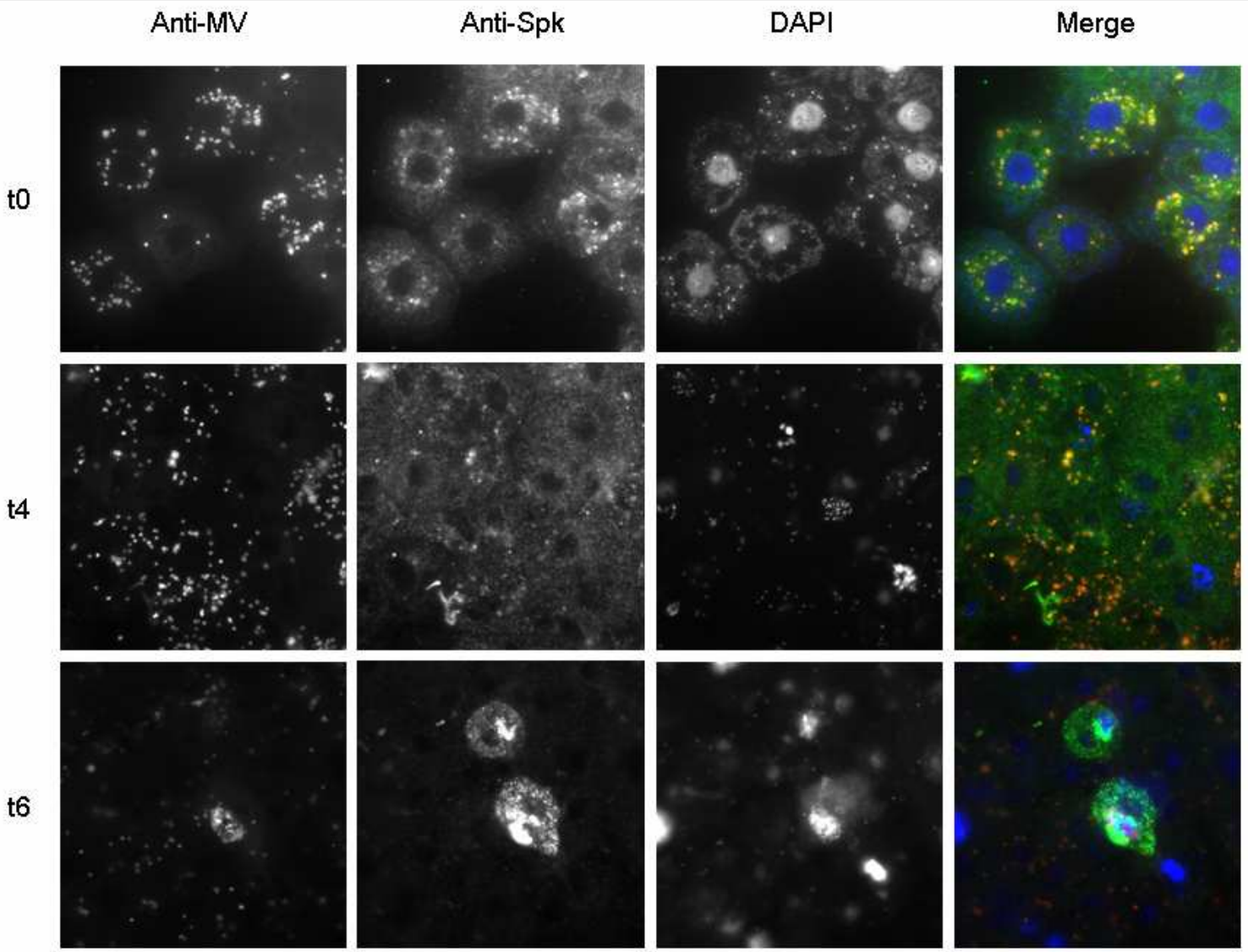
ORF_MAEO_YP_001         -----------KGTWDRGLDF----DKTYNLFVEEIERIKETGLKYKKDI---KSIAYLI
ORF_MJ_NP_24775         -----------SGTWDKGIDY----KQTYKMFKEDLQKLKNKELLYEDDY---KRIAYLI
ORF_TK_YP_18251         -----------PFAWDKGLDY----EKTYRQILNHYRQATRD-----------TSKAYDI
ORF_ATTV_YP_319         KILKPLGKGKIKGTWDYSVDF----VEAKKTLVSAYKQLYDTNLM--------PYEAYVA
ORF_STSV1_YP_07         ----------------------------RKTLEKDYKQFSQNSTE---DF---DKEIYVA
ORF_10_virophag         ------GAPQVAKGFDRGIDYLDIAAKLKKGLKKNYKVLQDTESTANAKRFAGSRVIYII
                                              . : .  .                    *

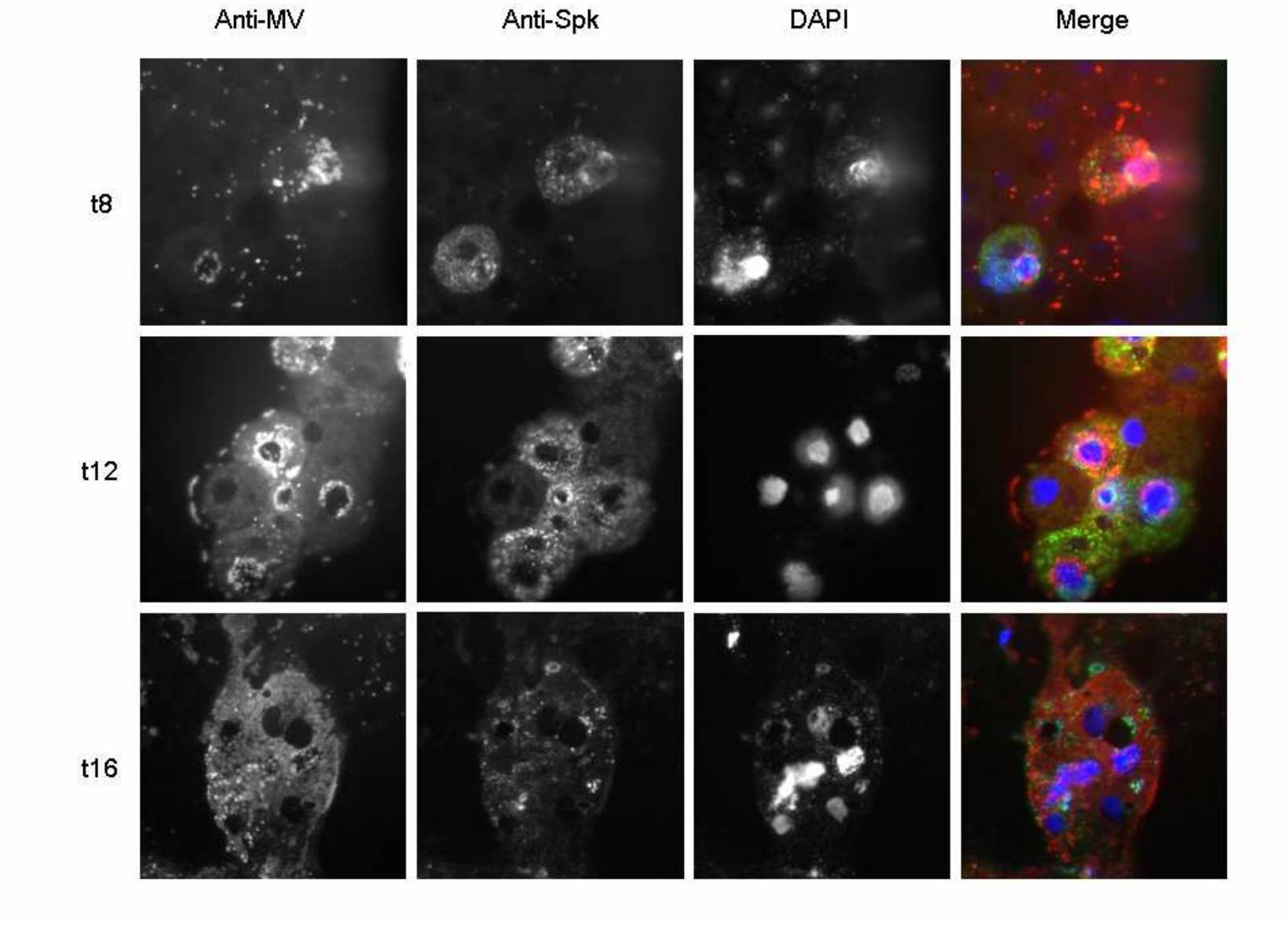
ORF_MAEO_YP_001         IALFQLRNGCRIGEAIEGILWICKNKDKINWNKPIEVS--VKVEKTKKSI----------
ORF_MJ_NP_24775         TFLFQLRNGCRIWEAIAGMINIAINIDNLNWNERITVK--VRTQK-RKDW----------
ORF_TK_YP_18251         ILLTQLRNGSRLTEAINFL-------KKLIEEKPFKRQKYIKVEK-RKDG----------
ORF_ATTV_YP_319         ILLIQLVNGCRIREAIRAF-------KTFIESGEREFQ--LQAQK---HG----------
ORF_STSV1_YP_07         ILLTQLLNGTRIGEAVKAF-------YQFVEVGGKERTIILKAEK---GG----------
ORF_10_virophag         IALLQLKNCSRISEAIVAT-------KKFSVSKNLNERVVVKIAKSEKDLIDRKTKDKIH
                          * ** *  *: **:            :           ::  *

ORF_MAEO_YP_001         ---AYRDMYFP-SMVKKEDIELIRGVILD--------------LENAKKPRMRIYQWLRN
ORF_MJ_NP_24775         ---EFRELIIP-KCIKKEDIEMVRDVFLDIKKEIDEKLTMDEKLKAKKKIVKRFGAWLYK
ORF_TK_YP_18251         ---YERLMVLP-EEIDKKELMRVSYV-----------------IKEANK--WKVSTYCKR
ORF_ATTV_YP_319         ---NIRFMIIP-DVVKKKATYNAVLTIDD-----------------EKLSARIRMFALH
ORF_STSV1_YP_07         ---NERKIIIP-KIITYKKYYSIILT-----------------KSERKMIAAVKMFCKR
ORF_10_virophag         TKPRYRDMVFPVDLVDTKIFKYIVKT------------KYWTKFCEFDSPRKRVLDFLLG
                             * : :* . :  :       .                    .    .  :

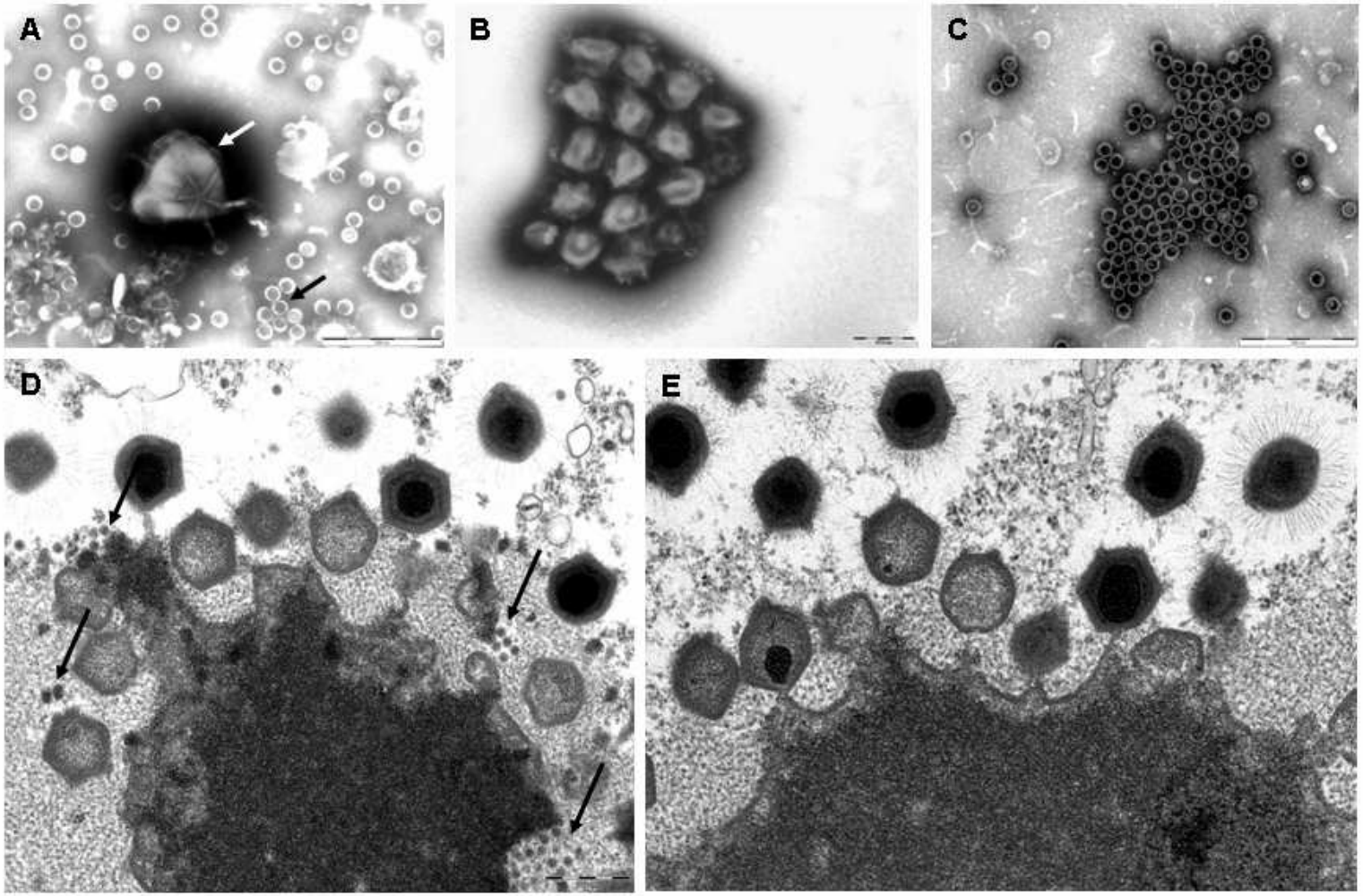
ORF_MAEO_YP_001         HYGINTHSLRYAFITKFAE-MNTSPQLIAKMTGHVNLNHLITYTQEKTANRMLRDLEIHITR
ORF_MJ_NP_24775         NYGINTHSLRYAYVTYLGE-HGIPAQVLAKITKHKNINYIETYTQSRLAKEILKNIG-DLDD
ORF_TK_YP_18251         TYGFNTHALRYALISYLSQ-KGVAPQLIAKITGHKTLDYILYYTQQQKAEELLKDL-----R
ORF_ATTV_YP_319         YLKANTHSLRYALISYLAK-NGIDPAIIAKITGHKRLDRIITYTQTKDAIEMLRKLA----D
ORF_STSV1_YP_07         RYGVNTHSFRYAFITKAIK-DGLPAEVVAKITGHKSLRHILTYVQTKEAEDYLARIA---NS
ORF_10_virophag         HYECNTHSLRYAGINYLLNVEKRDMNVVAKFVGHANVNQLVLYTQTKALDEIFDR---KIVV
                            ***::*** :.   :       ::**:. *  :  :  *.* :     :
```

Supplementary Figure 8. Alignment of the putative integrase (ORF 10). The virophage integrase, like all 130 members of the tyrosine recombinase family, contains the invariant RHRY amino-acid tetrad (grey boxes) in the C-terminal part of the protein.

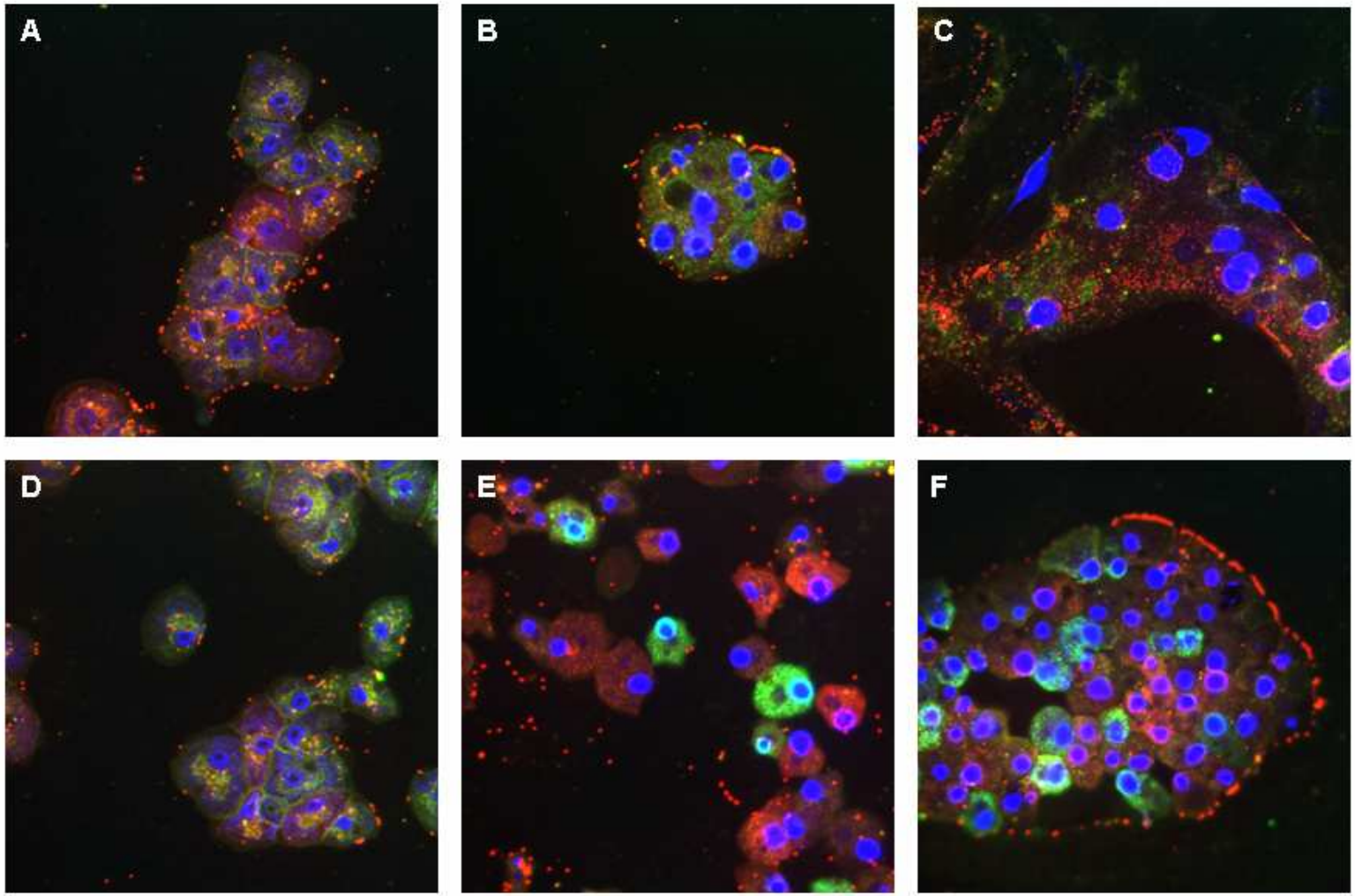

Anti-MV
Anti-Spk
DAPI
Merge
t0
t4
t6



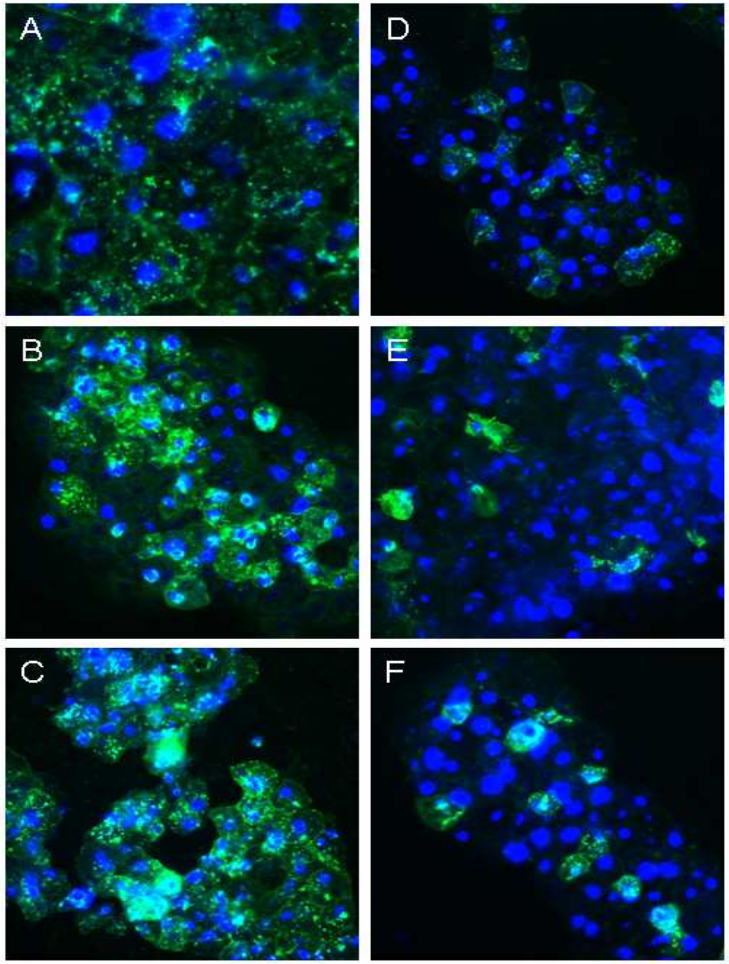
Supplementary Figure 9. *Mamavirus* and *Sputnik* replication cycle in co-infected *A. castellanii*. Viruses were stained with rabbit anti-*Mimivirus* plus goat anti-rabbit Ig-Alexa546 (anti-MV); and mouse anti-*Sputnik* (anti-Spk) plus goat anti-mouse Ig-FITC; VF were stained with DAPI.



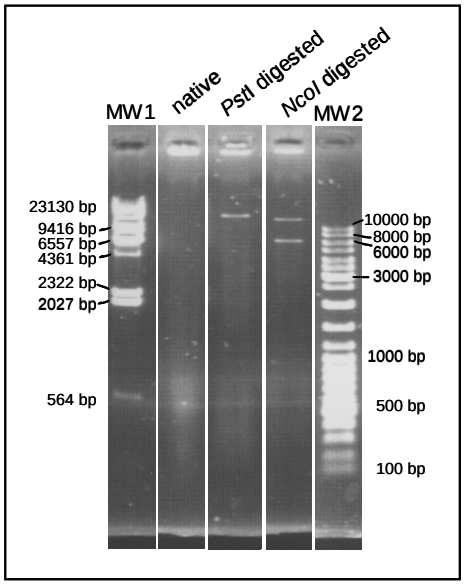
Supplementary Figure 10. Negative staining of viral supernatants containing (A) both *Mamavirus* (white arrow) and *Sputnik* (black arrow) (A), (B) purified *Mamavirus* and (C) purified *Sputnik*. Transmission electron pictures of *A. castellanii* infected (D, E) with the viral supernatant shown in (A) or in (B), respectively.



Supplementary Figure 11. Multiplication cycle of *Mamavirus* in the absence (A-C) or in the presence of *Sputnik* (D-F). Pictures were taken at 2h p.i. (A,D); 6h p.i. (B, E) or 8h p.i. (C, F).Viruses were stained with rabbit anti-*Mimivirus* plus goat anti-rabbit Ig-Alexa546 (anti-MV); and mouse anti-*Sputnik* (anti-Spk) plus goat anti-mouse Ig-FITC; VF were stained with DAPI.



Supplementary Figure 12. Comparison of *Sputnik* propagation in *Mamavirus*- versus *Mimivirus*-infected amoebae. *A. castellanii* were infected with a mixture of *Mamavirus* (A-C) or *Mimivirus* (D-F) and Sputnik. B and E, second round of infection; C and F, third round of infection. Sputnik was stained with mouse anti-Sputnik (anti-Spk) plus goat anti-mouse Ig-FITC; virus factories were stained with DAPI



Supplementary Figure 13. Agarose gel of the Sputnik native genome and the Sputnik genome after enzymatic digestion with *Pst*I and *Nco*I. Theoretical pattern of a circular genome digestion (*i.e.* one linear genome of 18343 bp and 2 bands of 5323 bp and 13020 bp respectively with *Pst*I and *Nco*I digestion) were respected. MW1: Lambda Hind III marker, MW2: GeneRuler DNA ladder (Fermentas).

2. SUPPLEMENTARY TABLES

Supplementary Table 1: primers used in this study

Used for	Target	Primer Name	Sense	Sequence (5' → 3')	Fragment size
Detection	Sputnik ORF 20 (major capsid protein)	ORF20-F ORF20-R	Forward Reverse	GAGATGCTGATGGAGCCAAT CATCCCACAAGAAAGGAGGA	174 bp
Circularization	Sputnik flanking regions	Spu-circ-F Spu-cir-R	Forward Reverse	GGTCGGTAAATCGACACCTG ACCCACAATTAGGGCATTCA	870 bp

Supplementary Table 2: Effect of *Sputnik* on the viability of *Mamavirus* infected *A. castellanii*

	Cell concentration x 10^{-5}/ml		% of lysed cells at 24h
	2h	24h	
Uninfected	4	12.5	-
Mamavirus	3.95	0.98	92.16
Mamavirus+Sputnik	4.35	2.61	79.12

Supplementary Table 3: Homologs of Sputnik protein sequences in the *Mimivirus* (complete, accession number NC_006450) and *Mamavirus* (accession numbers provided in the table) genomes as detected by tBLASTn

***Sputnik* ORF6 (310 aa)**	***Mimivirus* MIMI_R196**	***Mamavirus (EU827539)***
- Percent Identity	42.11	69.04
- Hit overlap	114	239
- E-value	1.99 e-20	3.25 e-87
***Sputnik* ORF12 (152 aa)**	***Mimivirus* MIMI_R546**	***Mamavirus (EU827540)***
- Percent Identity	61.34	62,18
- Hit overlap	119	119
- E-value	1.50 e-39	6.79e-40
***Sputnik* ORF13 (779 aa)**	***Mimivirus* L206/207**	***Mamavirus (EU827541)***
- Percent Identity	21.28	19.68
- Hit overlap	329	493
- E-value	2.04 e-12	8.35 e-14

Supplementary Table 4: Ct values of Sputnik capsid product in the pellet and the supernatant of *A. castellanii* cultures infected with *Mimivirus* and Sputnik, *Mamavirus* and Sputnik and Sputnik alone. Pellet and supernatant were collected at 0h post-infection (p.i.) (after a washing step) and 22h p.i. C_T values were 32.16, 41.31 and 19.86 for the amoeba negative control, the water negative control and the positive control (Sputnik DNA), respectively.

	Pellet		Supernatant	
	C_T values at 0h p.i.	C_T values at 22h p.i.	C_T values at 0h p.i.	C_T values at 22h p.i.
Mimivirus + Sputnik	19.71	16.64	19.53	16.24
Mamavirus + Sputnik	18.05	16.73	21.05	15.56
Sputnik alone	18.78	22.04	19.20	18.15

3. SUPPLEMENTARY METHODS

3.1. Sample preparation for two dimensional gel electrophoresis

Sputnik was purified by ultracentrifugation through a 25% sucrose cushion (100,000 × *g* for 30 min, 4°C) then treated as previously described[28]. Briefly, viral pellet was washed twice with phosphate-buffered saline (PBS) in the presence of protease inhibitors (Complete; Roche, Mannheim, Germany). The resulting pellet was solubilized in 40 mM Tris-HCl, pH 7.5, supplemented with 2% (wt/vol) sodium dodecyl sulfate (SDS; Sigma-Aldrich) and 60 mM dithiothreitol (DTT), followed by 5 min of heating at 95°C. The insoluble fraction was removed by centrifugation (12,000 × *g*, 4°C, 20 min), and soluble proteins were precipitated using a PlusOne 2-D cleanup kit (GE Healthcare) to remove SDS. The final pellet was resuspended in solubilization buffer {7 M urea, 2 M thiourea, 4% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS)} and stored at −80°C until isoelectric focusing (IEF) was performed.

3.2. Two dimensional gel electrophoresis

Immobiline DryStrips (18 cm, pH 3 to 10; GE Healthcare) were rehydrated overnight using 350 µl rehydration buffer (7 M urea, 2 M thiourea, 4% [wt/vol] CHAPS, 60 mM DTT, 0.5% [vol/vol] Immobiline pH gradient (IPG) buffer [GE Healthcare]) containing 100 µg of solubilized Sputnik proteins, and IEF was carried out according to the manufacturer's protocol (IPGphor II, GE Healthcare). Before the second-dimension electrophoresis was performed, strips were equilibrated twice in 10 ml equilibration buffer (30% [vol/vol] glycerol, 2% [wt/vol] SDS, 6 M urea, 50 mM Tris-HCl, bromophenol blue, pH 8.8) for 15 min. This buffer was supplemented with 65 mM DTT for the first equilibration and with 100 mM iodoacetamide for the second one. The strips were then embedded in 0.5% agarose, and the

proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Ettan DALT; GE Healthcare) at 5 W/gel for 30 min, followed by 4 to 5 h at 17 W/gel. Following migration, gels were then processed either by silver staining by a method compatible with mass spectrometry[29] or transferred onto nitrocellulose membranes in a semi-dry blotting apparatus (Semi-Phor unit, Hoefer Scientific Instruments, San Francisco, CA).

3.3. In-gel digestion and MALDI-TOF mass spectrometry

Spots excised from silver-stained gels were destained and subjected to in-gel digestion with trypsin[30] (sequencing-grade modified porcine trypsin; Promega, Madison, WI). Tryptic peptides were then extracted from the gel by successive treatments with 5% formic acid and 50% acetonitrile-5% formic acid. Extracts were pooled and dried in a Speedvac evaporator. Peptides resuspended in an α-cyano-4-hydroxycinnamic acid matrix solution (prepared by diluting 3 times a saturated solution in 50% acetonitrile-0.3% trifluoroacetic acid) were then spotted on the matrix-assisted laser desorption ionization (MALDI) target. Mass analyses were performed on a MALDI-time of flight (MALDI-TOF) Brüker Ultraflex spectrometer (Brüker Daltonique, Wissembourg, France). Mass spectra were internally calibrated using autolytic peptides from trypsin. Tryptic peptide mass lists were used to identify the proteins, using Mascot software (Matrix Science Ltd., London, United Kingdom). Sixty spots were analyzed, 1/60 was not identified and 2/60 were identified as corresponding to *Mimivirus* R135 encoded protein. Searches were performed against all available sequences in public databases and all possible open reading frames deduced from genomic data.

3.4. Western blotting

Nitrocellulose membranes were blocked in PBS supplemented with 0.2% Tween-20 and 5% non-fat dried milk (PBS-Tween-Milk) for 1.5 h before incubation with anti- Sputnik

sera previously adsorbed on *Mimivirus* and *Acanthamoeba castellanii* cell lysate and diluted 1:2,000 in PBS-Tween-milk. After 1 h incubation, membranes were washed three times with PBS-Tween and probed with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:1,000; GE Healthcare). Detection was achieved by chemiluminescence (ECL, GE Healthcare). Stained 2-D gels and immunoblot films were digitalized by transmission scanning (ImageScanner II, GE Healthcare). Lack of reaction with *Mimivirus* R135 encoded protein confirmed that adsorption by *Mimivirus* was efficient and that anti-Sputnik polyclonal antiserum was specific.

3.5. Inactivation of Sputnik and production of *Mamavirus*

A supernatant containing Sputnik and *Mamavirus* was submitted to heat-inactivation at 65°C or 60°C for 1 hour or to 48h desiccation. The viral suspension was then diluted in PAS by ten fold dilutions from 10^{-1} to 10^{-10}. Each dilution was inoculated onto 4 wells of a suspension of fresh amoebae and observed daily for lysis under inverted microscope. The supernatant of the last dilution producing lysis in 1/4 wells was sub-cultured onto fresh amoebae to produce *Mamavirus*. The absence of Sputnik was verified by transmission and negative staining electron microscopy, indirect immunofluorescence and Sputnik specific PCR.

3.6. Viral DNA extraction and PCR analysis

To verify the absence of Sputnik after the purification step, PCR were performed on the samples obtained above *i.e.*, “60°C -4 replicates”, “65°C -1 replicate”, and “desiccation - 4 replicates”. For each sample, DNAs extracted from 400 µl of the culture following the fluid/blood protocol of the QIAamp DNA Mini Kit (Qiagen, Valencia, California, United States). The PCR reaction mixture (25 µl total) contained target DNA, 1X Taq Buffer, 0.2

mM dNTPs, 1 µM each primer (Supplementary Table1), 1µl MgCl2, and 1U Taq DNA Polymerase. The thermocycler conditions were: 5 min at 95°C; 40 cycles of 1 min at 95 °C, 20 seconds at 57 °C, 1 min at 72°C; and 10 min at 72 °C. Amplification products were checked on a 1% agarose gel in TBE.

For Sputnik detection, real-time PCR targeting the Sputnik capsid gene (Supplementary table 1) were performed. Nucleic acids were extracted from a culture (pellet and supernatant 200 µl after centrifugation) of *A. castellanii* at 0h (a washed 30 minutes post-infection), and 22h post-infection with *Mimivirus* and Sputnik, *Mamavirus* and Sputnik or Sputnik alone. Amplifications were performed with the LightCycler FastStart DNA Master SYBR Green I™ kit in a standard PCR reaction as described by the manufacturer (Roche Diagnostics, Mannheim Germany). Each reaction contained 3 mM $MgCl_2$, 1 µM of each primer, and 5 µl of template DNA in a 20-µl PCR mixture. The amplification started with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 57 °C for 5 s, and 72 °C for 8 s with a temperature transition rate of 2 °C/s. Fluorescence signals were measured once in each cycle at the end of the extension step. After PCR amplification, T_m curve analysis was performed. For each samples, the C_T (Cycle Threshold) were given (Supplementary Table 4). The C_T corresponds to the PCR cycle number at which the fluorescence reaches a threshold value of 10 times the standard deviation of the baseline emission. The C_T values are inversely proportional to the starting amount of target DNA.

4. SUPPLEMENTARY RESULTS

4.1 Viral replication cycle

Sputnik and *Mamavirus* replication cycle in *A. castellanii*

A. castellanii were infected with a mixture of *Mamavirus* and Sputnik, as previously described for *Mimivirus*[4]. At different time points post-infection, cells were spotted on

microscope slides using a Cytospin. Indirect immunofluorescence labelling was performed using rabbit anti-*Mimivirus* serum and mouse anti-Sputnik serum, while nucleic acids were stained with DAPI. Results of *Mamavirus* and Sputnik co-infection are shown in Supplementary Figure 9. Numerous entering viruses were seen as bright dots in the cell cytoplasm at t0, which in fact corresponds to 30 min post-infection[4]. Cell nuclei were stained with DAPI. At 4h p.i. the first VFs were seen as distinct strongly stained patches in some cells, while the intensity labelling of the cell nuclei dropped. In these cells, no viral particles could be seen anymore, indicating an eclipse phase, as previously described for *Mimivirus*[2,4]. At 6h p.i. the VFs expanded and were homogenously strongly stained with DAPI. Sputnik production was detected from this time point and was localised as a bright green fluorescent polarised signal at one side of the VF. No *Mamavirus* was detected at this time. At 8h p.i. the VFs showed a diffuse fluorescent DAPI signal, while *Mamavirus* production could be observed around them. The late time points of infection (12h and 16h) revealed the huge production of *Mamavirus* accumulating into the cell cytoplasm. These observations allowed us to say that the two viruses were produced within the same VF with different kinetics and at different places, the Virophage being produced before *Mamavirus*. According to the genome sequence data, it might be hypothesised that expression of viral genes and proteins is strongly regulated within this new kind of VF.

Replication of Sputnik (comparison of 0h p.i. and 22h p.i.) was also confirmed by PCR (Supplementary Table 4).

Purification of *Acanthamoeba castellanii Mamavirus*

To obtain a pure suspension of *Mamavirus* we hypothesized that, as previously observed for *Mimivirus*[1], it would be resistant to high temperatures or desiccation. We thus submitted a supernatant containing Sputnik and *Mamavirus* to heat inactivation or dessication

to get rid of Sputnik particles. Compared to untreated supernatant, viral suspension treated at 60°C produced lysis in 4/4 infected wells until dilution 10^{-9}, in 1/4 wells until dilution 10^{-5} for viral suspension treated at 65°C, and in 4/4 wells until dilution 10^{-6} for viral suspension treated by desiccation. To verify the absence of Sputnik in these supernatants, PCR primers were designed based on the sequence coding the major capsid protein (ORF 20) (Supplementary Table 1, Supplementary Methods). No PCR product was obtained further confirming that *Acanthamoeba castellanii Mamavirus* was successfully purified from Sputnik particles. Untreated supernatant containing *Mamavirus* and Sputnik viruses and the 65°C-treated suspension were also analysed by negative staining electron microscopy staining to check for *Mamavirus* purity (Supplementary Figure 10 A and B respectively). The supernatant of the culture well lysed upon infection with the 10^{-5} dilution of the 65°C-treated suspension was then subcultured on *A. castellanii* for production of Sputnik-free *Mamavirus*.

Purification of Sputnik particles

Sputnik particles were purified by filtration through a 0.2 µm membrane as described in the Method Summary and On-line Method sections. Absence of *Mamavirus* in the preparation was assessed by negative staining electron microscopy (Supplementary Figure 10 C). Both *Sputnik*-free *Mamavirus* and *Mamavirus*-free Sputnik supernatants were then used to infect *A. castellanii*. At 16h p.i., *Mamavirus* VF and Sputnik could be seen in amoebae infected with a mixture of *Mamavirus* and Sputnik (Supplementary Figure 10 D), whereas only *Mamavirus* VF was visible upon infection with Sputnik-free *Mamavirus* (Supplementary Figure 10 E). No Sputnik particles could be evidenced by TEM examination of amoebae infected with *Mamavirus*-free Sputnik supernatant and no increased in the Sputnik production was observed by PCR (Supplementary Table 4). These results demonstrated that Sputnik

alone is a non infectious virus and that co-infection with *Mamavirus* is required for its multiplication cycle.

Co-infection of *A. castellanii* with purified *Mamavirus* and Sputnik

In order to evaluate the effect of Sputnik on the multiplication cycle of *Mamavirus*, *A. castellanii* were infected with either Sputnik-free *Mamavirus* or with the same amount of Sputnik-free *Mamavirus* pelleted by centrifugation and resuspended in a *Mamavirus*-free Sputnik supernatant. Infected cells were spotted on slides at different time p.i. and analysed by indirect immunofluorescence. Besides the presence of Sputnik virus in the co-infected cells (Supplementary Figure 11 D-F) compared to Sputnik-free *Mamavirus* infected cells (Supplementary Figure 11 A-C), no major difference could be seen between both cultures at 2, 6 or 8h p.i. or even later (not shown).

However an effect of Sputnik on the viability of *Mamavirus* infected cells was detected. Results are shown in Supplementary Table 2. After 24h of culture, about 92.2% of cells were lysed upon infection with Sputnik-free *Mamavirus* compared to about 79.1% of lysis due to *Mamavirus* and Sputnik co-infection. Thus, the presence of *Sputnik* resulted in about 13% reduction in *Mamavirus*-induced cell lysis. An effect of Sputnik on *Mamavirus* infectious titer was observed as well. End-point limiting dilution experiments showed that Sputnik-free *Mamavirus* supernatant induced amoebae lysis up to dilution $4.39\ 10^{-6}$, whereas *Mamavirus* and Sputnik supernatant induced lysis up to dilution $8.66\ 10^{-5}$. Detection of *Mamavirus* DNA by PCR confirmed the microscopic observations (data not shown).

Co-infection of *A. castellanii* with *Mimivirus* and Sputnik

Co-infection of *A. castellanii* with *Mimivirus* and Sputnik resulted in the same kinetics of viruses development cycles (data not shown), but the main difference was the percentage of

co-infected cells that is significantly lower in the case of *Mimivirus* and Sputnik than *Mamavirus* and Sputnik (Supplementary Figure 12 D and A, respectively). Viral supernatants taken at 24h were used to infect fresh amoebae cultures, and this was repeated twice in order to evaluate the evolution of the percentage of co-infected cells. Pictures taken at 16h p.i. after the second (Supplementary Figure 12 E and B) or the third round of infection (Supplementary Figure 12 F and C) showed no difference with the first one, suggesting a lower affinity of Sputnik for *Mimivirus* than for *Mamavirus*.

Replication of Sputnik (comparison of 0h p.i. and 22h p.i.) was also confirmed by PCR (Supplementary Table 4).

4.2 Sputnik genome analysis

Linearization of the Sputnik genome

A PCR primer set (Supplementary Table 1) has been designed on each extremities of the linear genome and amplification has been successfully performed. The PCR reaction mixture (25 µl total) contained target DNA, 1X Taq Buffer, 0.2 mM dNTPs, 1 µM each primer, 1µl MgCl2, and 1U Taq DNA Polymerase. The thermocycler conditions were: 5 min at 95°C; 35 cycles of 1 min at 95 °C, 1 minute at 60 °C, 1 min at 72°C; and 10 min at 72 °C. Amplification products were checked on a 1% agarose gel in TBE and sequenced. PCR product sequences allowed the circularization of the Sputnik's genome.

Around 50 ng of Sputnik DNA was digested with *Pst*I (Takara) and *Nco*I (Biolabs) following the manufacturer's protocol. One band, around 18 kb of molecular weight, was observed in a 0.6 % agarose gel (Supplementary Figure 13) with *Pst*I digestion while two bands around 5-6 kb and above 10 kb were observed for *Nco*I digestion. These confirmed the theoretical pattern of a circular genome digestion (*i.e.* one linear genome of 18343 bp and 2

bands of 5323 bp and 13020 bp respectively with *Pst*I and *Nco*I digestion), and the circular topology of the Sputnik genome.

Integration of Sputnik

There are some evidences showing that Sputnik is probably not integrated into the *Mamavirus* genome. First, even though the *Mamavirus* and Sputnik genomes were sequenced simultaneously, no read (over 20 Mbp of sequences) was found to overlap the two genomes. Second, integration into host chromosome usually occurs within specific tRNA genes. However, no such specific sites for integration have been found in *Acanthamoeba castellani* or in *Mamavirus* which possesses six tRNA genes (data not shown).

References

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