Ebola Virus VP40 Modulates Cell Cycle and Biogenesis of Extracellular Vesicles

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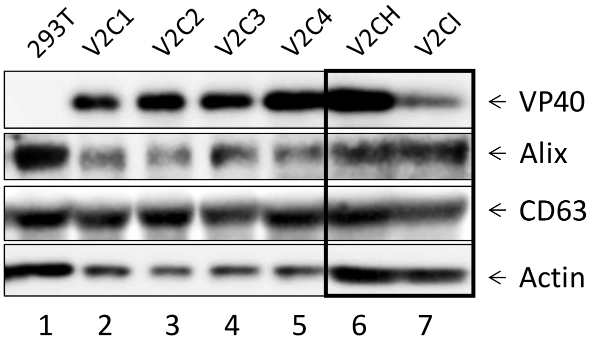
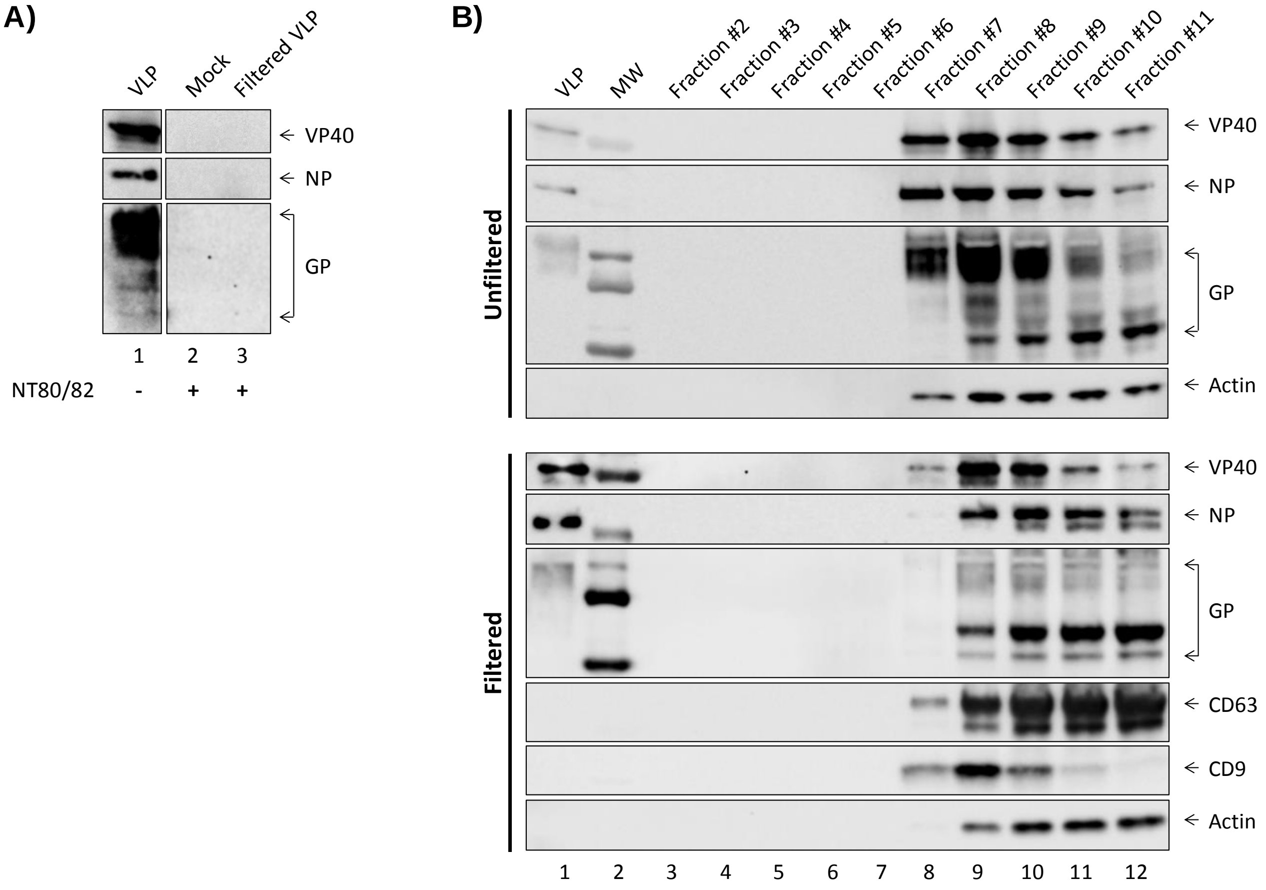
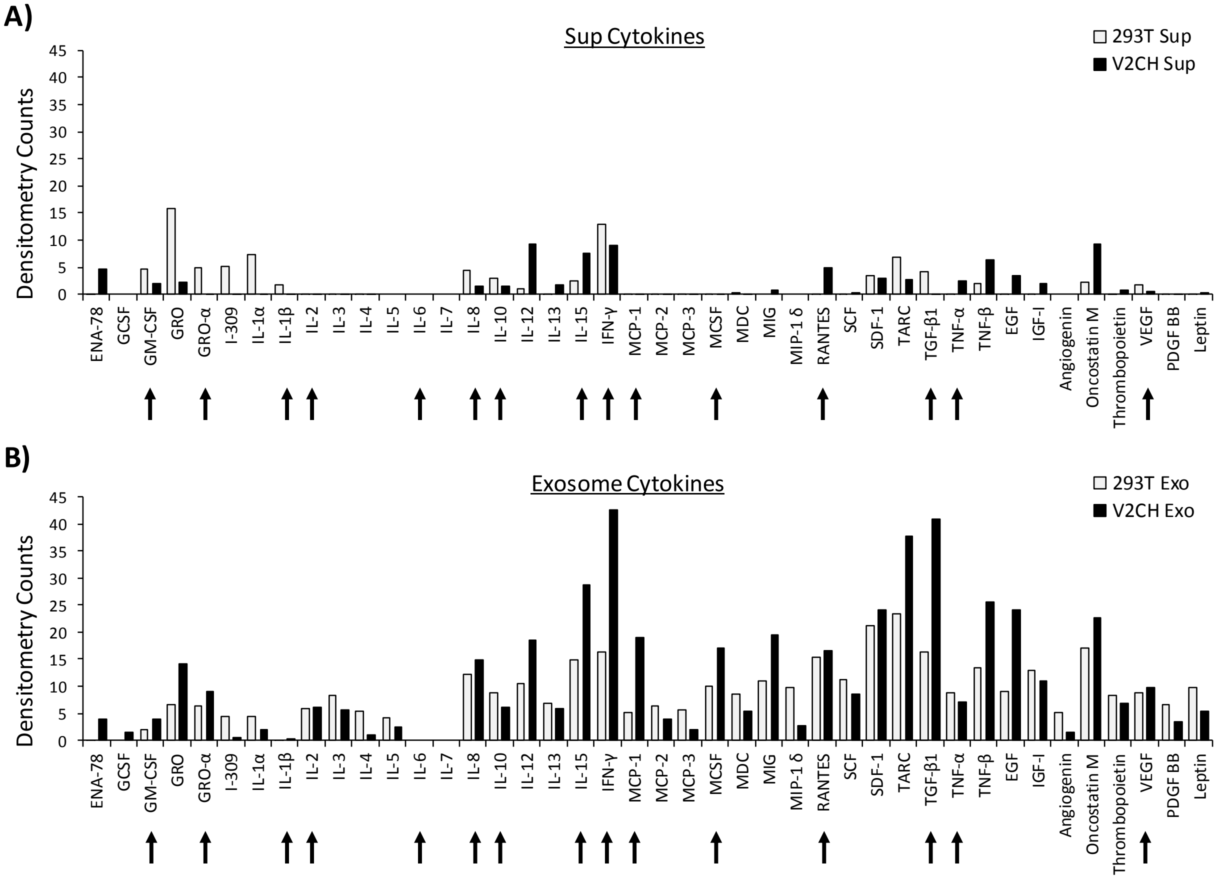


Supplementary Figure 1: Generation of stable VP40-producing cell lines. 293T cells were transfected with 20 µg of CMV-VP40 plasmid with antibiotic selection marker (pcDNA3.1/Hygro) by electroporation. Cells were treated next day with hygromycin B for plasmid selection (200-400 µg/mL). To generate VP40 clones, transfected cells were cultured for > 3 wk, followed by isolation of surviving colonies and multiple passages under specific antibiotic selection. Several of the resulting clones (V2C1-4, V2CH and V2CI) and control 293T cells were cultured for 5 days in exosome-free media, followed by harvesting and filtering (0.22 µm) of the supernatant. One milliliter of each supernatant was incubated with 30 µL NT80/82 particles for 72 h at 4°C. NT pellets were washed once in 1x PBS, followed by resuspension in

12 μL Laemmli buffer and loading onto a 4-20% Tris-glycine gel, and subsequent western blot analysis for VP40, exosomal markers Alix and CD63, and Actin protein levels. V2C cells chosen for future experiments (V2CH and V2CI) are enclosed by a black box.



Supplementary Figure 2: Effect of filtration on VLP. A) Five micrograms of VLPs were added into 1 mL of sterile 1x PBS and filtered (through 0.22 µm) alongside 1 mL of 1x PBS alone (mock). Filtered samples were incubated with 30 µL of NT80/82 particles (30% slurry) overnight at 4ºC, followed by processing and loading onto a 4-20% Tris-glycine gel, and subsequent western blot analysis for VP40, NP, and GP protein levels. Unfiltered VLP (0.25 µg) was loaded side-by-side with filtered samples. Selected lanes were taken from the same blot with identical exposure settings presented in the figure. **B**) V2CH cells were grown for 5 days in exosome-free media, followed by harvesting of supernatants. Supernatants were spiked with 10 µg of VLP and either left unfiltered or filtered (through 0.22 µm). VLP-spiked supernatants were then incubated with ExoMAX^{TM} (1:1 reagent:supernatant) overnight at 4ºC. EVs were pelleted, resuspended in 0.5 mL 1x PBS, and loaded on qEV columns. Fractions #2-11 (0.5 mL each) were collected and separately incubated with 30 µL NT80/82 at 4ºC for 24 h. EV-bound NTs were then processed for western blot analysis of VP40, NP, GP, CD63, CD9, and Actin levels. VLP (0.25 µg) positive control was loaded. MW = molecular weight ladder.



Supplementary Figure 3: Cytokine profiles of supernatants and exosomes from VP40-producing cells. 293T and V2CH cells were cultured for 5 d in exosome-free media, followed by harvesting of the supernatants, filtering (0.22 µm), and preparation for cytokine array assay. **A)** One milliliter of supernatant (Sup) from each cell type was used alongside 1 mL of filtered (0.22 µm) exosome-free DMEM media for incubation on human cytokine arrays. **B)** An additional 10 mL of supernatant samples from each cell type was incubated with 10 mL ExoMAX^{TM} (1:1 ratio sup:reagent) for EV precipitation overnight at 4ºC. The resulting EV pellet was resuspended in 300 µL sterile 1x PBS and loaded onto a 6-18% iodixanol density gradient (1.2% increments). Samples were ultracentrifuged at 100,000 x g for 90 min and fractions 10.8 and 12.0 were harvested and pooled (2 mL total). One milliliter of pooled exosome fractions were used side-by-side with sup and exosome-free DMEM samples for human cytokine array analysis. Exposures between membranes were matched according to positive and negative control signals. Raw densitometry counts for all membranes were obtained and normalized by subtracting the background signals represented by the negative control on each membrane. The background resulting from exosome-free DMEM media was subtracted from the signals produced by 293T and V2CH samples, and positive signals were graphed. Cytokines of significance for EBOV pathogenesis are indicated by black arrows.

Supplementary Table 1: Proteins of interest present in EVs from 293T and VP40-producing cells. 293T, V2CL, and V2CH cells were cultured for 5 days in exosome-free media, followed by harvesting and filtering (0.22 µm) of the supernatants. Ten milliliters of filtered supernatants were incubated with 10 mL ExoMAX^{TM} overnight at 4ºC. The resulting EV pellet was resuspended in 300 µL 1x PBS and loaded onto a 6-18% iodixanol density gradient (1.2% fraction increments). Samples were ultracentrifuged for 90 min at 100,000 x *g*, followed by collection of the 10.8 and 12.0 exosome fractions (1 mL each). Each fraction was incubated with 30 µL of NT80/82 particles at 4ºC for 48 h. Next, NT pellets were washed once with 1x PBS and processed for mass spectrometry. The data accumulated from the 10.8 and 12.0 fractions for each cell type were added and compared side-by-side to other cell types. Select proteins belonging to several categories are shown, along with their score and peptide hit number. Scores and peptide numbers correspond to summed 10.8 and 12.0 fractions. Cytokine-related proteins of interest were bolded.

Vesicle-Associated

Protein	V2CH		V2CL		293T	
	Score	**# Peptides**	**Score**	**# Peptides**	**Score**	**# Peptides**
heat shock protein HSP 90-beta isoform a	57.47	14	89.75	22	30.34	10
heat shock 70 kDa protein 6	6.70	4	11.26	3	8.11	3
heat shock cognate 71 kDa protein isoform 2	91.55	22	69.51	20	43.18	13
clathrin heavy chain 2 isoform 1	6.78	2	3.96	1	0.00	0
AP-1 complex subunit beta-1 isoform b	6.19	2	2.90	1	0.00	0
AP-2 complex subunit beta isoform b	6.19	2	2.90	1	0.00	0
stress-induced-phosphoprotein 1 isoform b	9.42	3	7.61	2	0.00	0
vacuolar protein sorting-associated protein 35	9.31	3	3.00	1	0.00	0
clathrin heavy chain 1 isoform 2	22.21	6	3.96	1	2.63	1
coatomer subunit alpha isoform 1	19.80	6	0.00	0	0.00	0
synaptic vesicle membrane protein VAT-1 homolog	46.64	9	53.92	13	30.06	6

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heat shock cognate 71 kDa protein isoform 2	91.55	22	69.51	20	43.18	13
clathrin heavy chain 2 isoform 1	6.78	2	3.96	1	0.00	0
AP-1 complex subunit beta-1 isoform b	6.19	2	2.90	1	0.00	0
AP-2 complex subunit beta isoform b	6.19	2	2.90	1	0.00	0
stress-induced-phosphoprotein 1 isoform b	9.42	3	7.61	2	0.00	0
vacuolar protein sorting-associated protein 35	9.31	3	3.00	1	0.00	0
clathrin heavy chain 1 isoform 2	22.21	6	3.96	1	2.63	1
coatomer subunit alpha isoform 1	19.80	6	0.00	0	0.00	0
synaptic vesicle membrane protein VAT-1 homolog	46.64	9	53.92	13	30.06	6

Nuclear/Transcription-Associated

Protein	V2CH		V2CL		293T	
	Score	**# Peptides**	**Score**	**# Peptides**	**Score**	**# Peptides**
lamin isoform A	24.57	6	26.79	5	0.00	0
lamin isoform D	17.55	4	15.54	4	0.00	0
nucleolin	56.62	13	87.51	16	23.44	5
staphylococcal nuclease domain-containing protein 1	31.86	9	30.90	9	5.56	2
histone H1.1	16.68	3	30.34	4	0.00	0
histone H1.3	37.96	8	51.21	9	0.00	0
histone H1.4	37.96	8	51.21	9	0.00	0
histone H4	15.05	4	17.04	5	0.00	0
histone deacetylase 1	9.69	2	7.08	2	0.00	0
histone-binding protein RBBP7 isoform 2	5.38	3	2.50	1	0.00	0
DNA replication licensing factor MCM2	14.81	5	8.75	3	2.61	1
FACT complex subunit SSRP1	15.51	4	11.21	4	0.00	0
transcription elongation factor SPT6	7.72	4	0.00	0	0.00	0
protein arginine N-methyltransferase 1 isoform 3	15.99	3	10.38	3	0.00	0
non-POU domain-containing octamer-binding protein isoform 1	15.91	3	3.62	1	0.00	0
Y-box-binding protein 3 isoform b	15.76	3	23.51	3	0.00	0
replication protein A 70 kDa DNA-binding subunit	9.50	3	4.96	2	2.37	1

Cell Death/Proteasome

	V2CH		V2CL		293T	
Protein	**Score**	**# Peptides**	**Score**	**# Peptides**	**Score**	**# Peptides**
programmed cell death protein 6 isoform 2	0.00	0	5.49	2	0.00	0
proteasome activator complex subunit 3 isoform 1	32.87	6	11.69	2	0.00	0
nuclear protein localization protein 4 homolog	5.92	2	0.00	0	0.00	0

Protein Transport

	V2CH		V2CL		293T	
Protein	**Score**	**# Peptides**	**Score**	**# Peptides**	**Score**	**# Peptides**
importin-7	5.63	2	0.00	0	0.00	0
importin subunit beta-1 isoform 1	11.96	4	8.75	3	0.00	0
importin subunit beta-1 isoform 2	6.66	2	6.20	2	0.00	0
PREDICTED: importin-5 isoform X1	9.40	3	7.06	2	0.00	0

RNA Binding

	V2CH		V2CL		293T	
Protein	**Score**	**# Peptides**	**Score**	**# Peptides**	**Score**	**# Peptides**
heterogeneous nuclear ribonucleoprotein A1 isoform a	17.92	5	5.60	2	2.66	1
heterogeneous nuclear ribonucleoprotein A1-like 2	14.96	4	5.60	2	0.00	0
heterogeneous nuclear ribonucleoproteins A2/B1 isoform A2	22.99	5	2.82	1	0.00	0
heterogeneous nuclear ribonucleoprotein A/B isoform a	20.02	5	11.88	4	0.00	0
heterogeneous nuclear ribonucleoprotein A3	14.17	4	13.95	3	0.00	0
heterogeneous nuclear ribonucleoprotein D0 isoform d	16.30	4	24.02	7	0.00	0
heterogeneous nuclear ribonucleoprotein R isoform 3	13.20	5	10.14	4	0.00	0
heterogeneous nuclear ribonucleoprotein K isoform b	21.46	4	8.10	2	0.00	0
heterogeneous nuclear ribonucleoprotein L isoform a	13.82	4	0.00	0	0.00	0
heterogeneous nuclear ribonucleoprotein Q isoform 2	4.59	2	4.97	2	0.00	0
polyadenylate-binding protein 3	15.00	4	15.19	4	4.24	1
polyadenylate-binding protein 4 isoform 1	14.08	4	13.14	4	0.00	0
poly(rC)-binding protein 2 isoform e	9.41	4	2.59	1	0.00	0
probable ATP-dependent RNA helicase DDX17 isoform 3	13.80	4	3.18	1	0.00	0
probable ATP-dependent RNA helicase DDX6	11.49	4	2.87	2	0.00	0
ATP-dependent RNA helicase A	34.07	11	39.89	12	7.30	2
ELAV-like protein 1	15.12	3	3.17	1	0.00	0

RNA Splicing

	V2CH		V2CL		293T	
Protein	**Score**	**# Peptides**	**Score**	**# Peptides**	**Score**	**# Peptides**
splicing factor 3B subunit 3	5.02	3	6.92	2	0.00	0
cleavage and polyadenylation specificity factor subunit 5	10.28	2	8.99	3	0.00	0
nuclease-sensitive element-binding protein 1	15.76	3	23.51	3	0.00	0
small nuclear ribonucleoprotein Sm D1 isoform 1	22.26	5	22.05	5	3.80	1
spliceosome RNA helicase DDX39B	14.86	5	7.77	3	0.00	0
U5 small nuclear ribonucleoprotein 200 kDa helicase	22.17	7	3.20	1	0.00	0

Other

	V2CH		V2CL		293T	
Protein	**Score**	**# Peptides**	**Score**	**# Peptides**	**Score**	**# Peptides**
Golgi apparatus protein 1 isoform 2 precursor	31.55	9	52.66	11	5.31	2
ubiquitin-like modifier-activating enzyme 1	25.94	5	19.65	7	3.14	1
voltage-gated potassium channel subunit beta-2 isoform 2	12.38	3	11.54	3	0.00	0
T-complex protein 1 subunit epsilon	16.68	4	20.81	8	3.19	1
T-complex protein 1 subunit eta isoform c	11.69	4	15.67	4	0.00	0
catalase	6.30	2	0.00	0	0.00	0
peroxidasin homolog precursor	109.14	23	196.17	32	102.00	22
ATP-citrate synthase isoform 3	102.29	26	87.61	23	33.67	9
nucleoside diphosphate kinase A isoform b	28.84	6	37.20	8	7.65	2
metalloproteinase inhibitor 2 precursor	8.57	2	28.39	6	5.02	1
ras GTPase-activating-like protein IQGAP1	37.52	9	22.55	7	4.28	1
interleukin enhancer-binding factor 2 isoform 1	33.26	6	14.61	5	0.00	0
interleukin enhancer-binding factor 3 isoform c	19.25	5	14.92	3	0.00	0
proliferation-associated protein 2G4	6.19	2	5.57	2	0.00	0
vascular endothelial growth factor A isoform a	6.89	3	2.35	1	5.50	2
latent-transforming growth factor beta-binding protein 1 isoform 3 precursor	7.54	2	0.00	0	7.63	2
transforming growth factor beta-1 precursor	2.65	1	6.18	2	2.49	1
insulin-like growth factor-binding protein 4 precursor	8.22	1	9.12	2	0.00	0
insulin-like growth factor II isoform 2	4.14	1	3.77	1	0.00	0